

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)	
Mathias Alterman et al.)	Group Art Unit: 1626
Application No.: 10/721,892)	Examiner: Laura Lynne Stockton
Filed: November 26, 2003)	
For: Tricyclic Compounds Useful as)	Confirmation No.: 5982
Angiotensin II Agonists)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

I, THOMAS UNGER, declare as follows:

1. I studied medicine in Germany and the UK, and gained my M.D. from the University of Heidelberg, Germany in 1975. I then carried out postdoctoral research at the Clinical Research Institute of Montreal, Canada, and the Department of Pharmacology in Heidelberg, where I received my Ph.D. in Pharmacology in 1982. Prior to 1994, I held professorships in pharmacology and hypertension research at the University of Heidelberg. Between 1994 and 2001, I was Director of the Institute of Pharmacology at the University of Kiel, Germany. Between 2001 until 2006, I was Director of the Institute of Pharmacology and Toxicology, Campus Mitte of the Charité – Universitätsmedizin Berlin. I presently hold the Chair of Pharmacology and am Director of the Institute of Pharmacology at the Charité Berlin, as well as the Director of the Center for Cardiovascular Research (CCR) at the Charité, Berlin. I am also Chairman of the German Institute for High Blood Pressure Research in Heidelberg.
2. I am a member of the German Societies of Pharmacology, Cardiology and Hypertension (Council Member 1995–2001), the International Society of Hypertension, the European Society of Hypertension (Council Member 1989–97), the European Council for Blood Pressure and Cardiovascular Research (President,

2000–2) and the Inter-American Society of Hypertension. I am also a Fellow of the American Heart Association and was Chairman of the Angiotensin Gordon Research Conference in 1999. In recognition of my work in the hypertension field, I have received the following awards: The German Hypertension Society's Franz Gross Award for Hypertension Research (in 1989), the Meilahti Lecture Award of the Medical Faculty, University of Helsinki, Finland (in 1995), the Björn Folkow Award of the European Society of Hypertension (in 1999), and the Robert Tigerstedt Award of the Finnish Hypertension Society in 2005).

3. I have authored or co-authored more than 600 scientific publications. I am or have been a member of the Editorial Boards of the following journals: *American Journal of Physiology*, *Biochemical Pharmacology*, *Blood Pressure*, *Cardiovascular Drugs and Therapy*, *Clinical and Experimental Hypertension*, *Hypertension*, *Hypertension Research*, *Journal of Hypertension*, *Fundamental and Clinical Pharmacology*, *Physiological Genomics*, *Regulatory Peptides*, *High Blood Pressure & Cardiovascular Prevention*. I attach a list of my publications as Exhibit A. I believe that I am qualified to make this declaration.
4. I act as a consultant for the Assignee of the present application, although all remuneration received for consultancy services is paid to the Charité – Universitätsmedizin Berlin, and not directly to me. Otherwise, any interest that I have in the assignee is as set out at the end of Kaschina *et al* in *Circulation* (2008) 118, 2523–32 (copy attached as Exhibit B; see "Disclosures" section on last page).
5. I have read the specification of the present application (originally published as international patent application WO 02/096883; copy attached as Exhibit C), as well as the claims that are pending as of the date specified below. I note that the main claim as pending relates to a group of compounds and that, according to what is stated in the present application, these compounds are angiotensin II (AngII) agonists, and more particularly agonists of the AngII subtype 2 receptor (also known as the AT2 receptor; see page 1, lines 6 to 9 and page 26, lines 10–13 of Exhibit C in this regard). To my knowledge, the compounds disclosed in the present specification are the first ever small molecule (i.e. non-peptide) AT2 receptor-specific agonists that have been described.

6. AT₂ agonism is empirically demonstrated by way of Examples 14 and 15 of the application as filed. Example 15 in particular states that title compounds of the Examples of the application were tested in Test C (see Exhibit C at page 34, lines 2 to 7). This is a method described by Flemström *et al* in *Am. J. Physiol.* (1982) 243, G348-58 (copy attached as Exhibit D). Attenuation of the effect described in that assay by the known AT₂ receptor antagonist PD123319 (see Exhibit C, Example 15 at page 65) demonstrates that the positive effect observed (stimulation of duodenal mucosal alkaline secretion in barbiturate anaesthetised rats) must be due to agonism of the AT₂ receptor.
7. I have also read and understood the content of the pending Office Action in the present application as well as the prior art that has been cited by the Examiner. I note from the Office Action that the Examiner rejects the pending claims on the basis that they are allegedly obvious over European patent application EP 512 675 A1 (hereinafter "Allen *et al*"). The Examiner states that the main difference between the compounds of Allen *et al* and the instantly claimed compounds is that the latter comprise a hydrogen atom (at the 2-position of the imidazole ring) whereas the prior art compounds comprise a lower alkyl group at the same position, and that the substitution of a hydrogen atom for a lower alkyl is not a patentable modification (see, Office Action mailed May 25, 2007, at page 7).
8. Having read Allen *et al*, and in particular the assays described at page 45, line 57 to page 49, line 39, I note that the compounds disclosed therein are Angiotensin II receptor antagonists. Although it is not stated in Allen *et al* (presumably in view of the age of the document) which receptor subtype the compounds of that document bind to, in view of the screens and indications mentioned in that document, the suggestion is that it is AT₁. In any event, the compounds of Allen *et al* present quite different biological activity to the AT₂ agonist activity exhibited by the presently-claimed compounds.
9. I submit that there is nothing in the prior art (cited or otherwise) to suggest that making the modification referred to in the Office Action would lead not only to a switch from antagonism of a receptor to agonism of a receptor, but also to selective

agonism of a *different* receptor subtype. This in itself is entirely unexpected to me as a person of skill in the art.

10. The Examiner has also stated that Allen *et al* discloses that the compounds described therein may be useful in the treatment of hypertension and has pointed out that this is also one of the disease states mentioned in the instant specification (see Office Action mailed March 14, 2008, at pages 8 and 9). It is asserted by the Examiner that the Applicant is therefore claiming compounds that are useful for treating the "same" diseases/disorders that are taught by Allen *et al*. (see Office Action mailed March 14, 2008, at page 9). My reading of the Office Action is that the Examiner asserts that the skilled person would "expect" that the compounds would be useful in the treatment of, for example, hypertension, and that she wants to see a persuasive side-by-side showing of unexpected, beneficial and/or superior results for the presently claimed compounds over Allen *et al*.
11. Although I shall deal with this aspect of the Examiner's rejection later, I firstly think that it is necessary for me to provide a detailed explanation of why the unexpected difference in biological activity, based upon what is known in the art, is likely to make a huge difference in the clinical setting and that, accordingly, the noted change in activity gives rise to beneficial and superior results.
12. Drugs that bind to physiological receptors and mimic the effect of the natural, endogenous ligand are termed agonists, whereas those that bind to receptors and do not mimic, but interfere with the binding of the endogenous ligand are termed antagonists. Pure antagonists are themselves devoid of any intrinsic (receptor stimulating) activity, but they produce effects by inhibiting the action of the natural ligand by competition for the ligand binding sites. (See, for example, E. M. Ross. *Pharmacodynamics: Mechanisms of drug action and the relationship between drug concentration and effect* in: J. G. Hardman *et al* (eds.): *Goodman & Gilman's - The Pharmacological Basis of Therapeutics*, 9th Ed., McGraw-Hill, New York, 1996, at page 30, left column; copy attached as Exhibit E).
13. As stated in Exhibit C at page 1, line 16 to page 2, line 10, AngII is an endogenous hormone that is a highly active component of the renin-angiotensin system (RAS).

The RAS plays an important role in the regulation of *inter alia* blood pressure. Two main subtypes of AngII receptors (AT1 and AT2) have been identified, which appear to have opposing effects.

14. AngII is a potent vasoconstrictor. When AngII is present in unphysiologically high amounts, vasoconstriction is caused by stimulation of the AT1 receptor by AngII (de Gasparo *et al*, *Pharmacol. Rev.*, 2000, 52, 415-72, enclosed as Exhibit F; see the Introduction). This results in increased blood pressure (i.e., hypertension), which in turn promotes inflammation and fibrosis, and may lead to conditions such as hypertensive nephropathy and left ventricular hypertrophy (see Exhibit F at, for example, page 429).
15. Thus, AT1 receptor antagonists (which are well known in the art to include drugs such as losartan, telmisartan, irbesartan, olmesartan and valsartan) act by blockade of the AT1 receptor and therefore compete with AngII, resulting in a lowering of blood pressure by way of a vasodilatory effect (see, for example, Exhibit F at pages 427 and 428). In this way, the pathological chain of events that is initiated by AngII binding to the AT1 receptor is interrupted, and AT1 receptor antagonists are capable of counteracting the detrimental effects of overproduction of AngII, such as hypertension, inflammation, and fibrosis.
16. On the other hand, stimulation of the AT2 receptor with an AT2 receptor agonist will have no effect on the binding of AngII to the AT1 receptor. AT2 receptor stimulation is instead known to have an effect on pathological chains of events occurring downstream of various receptors (not just the AT1 receptor) by interaction with second messenger cascades (see Exhibit F at pages 440 to 446, as well as the more recent review article by Steckelings *et al* in *Peptides*, 2005, 26, 1401-09, attached as Exhibit G). In this respect, AT2 agonism is likely to give rise to a much broader and clinically beneficial range of activity than is available from treatment with AT1 receptor antagonists.
17. There is recent evidence to suggest that, unlike AT1 receptor antagonists, AT2 receptor agonists do not have a direct effect on the lowering of blood pressure and therefore hypertension (directly). I refer in this regard to Exhibit B (see Table 1).

Instead, AT2 receptor agonists, such as those presently claimed are more likely to have a direct positive and more beneficial effect on the *consequences* of hypertension, such as inflammation, hypertrophy, and fibrosis, as mentioned above.

18. To elucidate, it is well known that inflammatory responses are mainly induced by cytokines or chemokines (see Whiteside, *Introduction to Cytokines as Targets for Immunomodulation*, in House and Descotes (eds.). *Cytokines In Human Health: Immunotoxicology, Pathology, and Therapeutic Applications*, Humanapress, Totowa, New Jersey; 1st edition (2007); copy attached as Exhibit H). Therefore, an increased synthesis of cytokines and chemokines is a substantial part of an inflammatory reaction. Synthesis of most cytokines and chemokines is promoted by the nuclear transcription factor NF- κ B (see Ghosh and Hayden, *Nature Reviews Immunology*, 2008, 8, 837-48; copy attached as Exhibit I). Stimulation of NF- κ B activity is also part of the inflammatory response initiated by AngII via the AT1 receptor (see, for example, Skurk *et al*, in *Arterioscler. Thromb. Vasc. Biol.*, 2004, 24, 1199-203; copy attached as Exhibit J).
19. Unlike AT1 receptor antagonists, AT2 receptor agonists are capable of directly inhibiting NF- κ B activity (see, for example, Wu *et al*, *Molecular Endocrinology*, 18, 666-78 (2004); copy attached as Exhibit K). In particular, AT2 receptor agonists counteract NF- κ B activation by conventional cytokines, such as tumor necrosis factor- α (TNF α). In this way, the anti-inflammatory effect of AT2 receptor stimulation is broader than the anti-inflammatory effect of the AT1 receptor blockade, because AT1 receptor blockade is restricted to an attenuation of AngII-induced inflammation, unlike AT2 receptor stimulation. This is likely to give rise to a much more potent anti-inflammatory response in the clinic.
20. Further, induction of hypertrophy and fibrosis by AngII and other growth factors is mediated by a cascade of intracellular phosphorylations exerted by various kinases. Unlike AT1 receptor antagonists, AT2 receptor agonists stimulate the activity of phosphatases, i.e., of enzymes catalysing dephosphorylation (see, for example, attached Exhibit G). Such phosphatases are then able to interfere with all kinds of signalling cascades that involve kinases. Thus, the range of effectiveness of AT2 receptor agonists against hypertrophy and fibrosis is broader than is the case for AT1

receptor antagonists because the AT2 receptor agonist's mode of action is not restricted to phosphorylation cascades activated by AngII, but is also involved in phosphorylation cascades induced by conventional growth factors.

21. Finally, and equally importantly, as stated in Exhibit C at *inter alia* page 2, lines 12 to 29, the AT2 receptor has been shown to be involved in apoptosis, inhibition of cell proliferation and to play a beneficial role in tissue injuries, such as vascular injury, wounds, dyspepsia, irritable bowel syndrome and multiple organ failure. AT2 receptor agonists are therefore of potential utility in some disease states that simply cannot be treated by AT1 receptor antagonists.
22. In summary, potential AT2 receptor agonists are likely to have a much broader range of activity in the clinic than AT1 receptor antagonists for at least the following reasons. AT2 receptor agonists potentially:
 - (a) are useful in the *direct* healing of organic diseases related to conditions such as hypertension;
 - (b) give rise to a much broader range of anti-inflammatory and anti-fibrotic activity than is possible with AT1 receptor antagonists; and
 - (c) are useful in the treatment of diseases that cannot be treated by simple blockade of the AT1 receptor.
23. With reference to paragraph 10 above, I understand that the Examiner has expressed an interest in seeing a side-by-side comparison between a compound from Allen *et al* that is specifically identified by the Examiner as Compound 51 (see page 65 of Allen *et al*) and the compounds of the present application.
24. I received Compound 51 from the Assignee during November 2008 and was asked to confirm that it is, as is stated in Allen *et al*, an AngII AT1 subtype receptor antagonist. I understand that the compound was synthesised under contract on behalf of the Applicant. The experiments set forth hereinafter were carried out under my supervision.
25. Human primary dermal fibroblasts, which express both the AT1 and AT2 AngII receptor subtypes, were seeded in 6-well plates and grown to 80% confluence. Prior

- to stimulation, cells were serum-starved for 24 hours. Interleukin-6 (IL-6) was used as an inflammatory marker and IL-6 mRNA expression was quantified by real-time RT-PCR after 10 hours of stimulation. A control group, and a vehicle only (DMSO) group, were employed for comparison.
26. To verify the pro-inflammatory effect of AngII, cells were stimulated with AngII (100 nM in DMSO) with or without pre-treatment with the known AT1 receptor antagonist irbesartan (10 μ M in DMSO; hereinafter "Irb"; Sanofi-Aventis) for 30 minutes. The results are shown in the attached Fig. 1B (results designated "Ang II" and "Ang II+Irb", respectively). As shown in this figure, Irb attenuates the pro-inflammatory effect of AngII.
27. A similar experiment was carried out with Compound 51 of Allen *et al* (referred to as "UNV" hereinafter and in the attached figures). When cells were pre-incubated with UNV (1 μ M in DMSO) for 30 minutes before adding AngII, the pro-inflammatory effect of AngII was also attenuated (see Fig. 1A; compare "Ang II" sample to "Ang+UNV" sample, $p < 0.02$). This suggests that UNV is acting in the same way as Irb (i.e., as stated in Allen *et al*, it is an AngII antagonist). In order to demonstrate that this inhibition is mainly due to AT1 sub-receptor blockade and not AT2 receptor agonism, fibroblasts were first pre-incubated with the selective AT2 receptor antagonist PD123319 (10 μ M in DMSO; Sigma; see paragraph 6 above) for 30 minutes, followed by incubation with UNV and with AngII. As shown by the result designated "AngII+UNV+PD" of Fig. 1A, the effect of UNV on AngII induced IL-6 mRNA expression was not affected by the presence of an AT2 receptor specific antagonist (compare "Ang+UNV" to "AngII+UNV+PD", $p > 0.35$). This demonstrates that the attenuation observed in Fig. 1A (i.e. that designated "Ang+UNV") is due to binding of UNV to the AT1 receptor. The results are collated in Fig. 1C.
28. As discussed at paragraph 6 above, the application as filed shows that compounds of the present application act as AT2 receptor agonists. In order to confirm this, and to show that UNV is not an AT2 receptor agonist, further experiments were performed in which cells were incubated with TNF α (10 ng/mL; designated TNF α in the attached figures) to augment IL-6 synthesis. I refer to paragraph 19 above as confirmation of the relevance of this experiment.

29. Fibroblasts were treated either with $\text{TNF}\alpha$ alone or, additionally, were co-incubated with the compound of Example 1 of the instant specification (1 μM ; called compound "C21" in the attached figures), which I understand is the elected species in the present application. The results are shown in Fig. 2B, where it can be seen that compound C21 produces a marked attenuation of the effect on IL-6 expression by $\text{TNF}\alpha$ (compare results designated " $\text{TNF}\alpha$ " and " $\text{TNF}\alpha + \text{C21}$ ", $p < 0.01$). Further, this effect was reversed completely when fibroblasts were pre-incubated with the selective AT2 receptor antagonist PD123319 (10 μM ; see Fig. 2B, compare " $\text{TNF}\alpha + \text{C21}$ " to " $\text{TNF}\alpha + \text{C21} + \text{PD}$ ", $p < 0.0003$) before incubation with compound C21 and $\text{TNF}\alpha$. This experiment confirms that compound C21 attenuates the effect of $\text{TNF}\alpha$ on IL-6 synthesis by agonism of the AT2 receptor and, accordingly, that compound C21 is an AT2 agonist.
30. A similar experiment was carried out with UNV (1 μM in DMSO). In contrast to the same experiment with compound C21, a marked *increase* in IL-6 expression was observed when the sample was co-incubated with UNV and $\text{TNF}\alpha$ (see Fig. 2A, compare results designated " $\text{TNF}\alpha$ " and " $\text{TNF}\alpha + \text{UNV}$ ", $p < 0.001$). Further, this effect was not affected when fibroblasts were pre-incubated with the selective AT2 receptor antagonist PD123319 (10 μM in DMSO) before incubation with UNV and $\text{TNF}\alpha$ (see Fig. 2A, compare " $\text{TNF}\alpha + \text{UNV}$ " to " $\text{TNF}\alpha + \text{UNV} + \text{PD}$ "). This experiment confirms that UNV is not acting as an AT2 agonist. The results are collated in Fig. 2C.
31. As noted above, Allen *et al* generally describes their compounds as AngII receptor antagonists. Based on assays provided in that reference, Allen's compounds appear to act as an AT1 receptor antagonist. The reference is silent on whether their compounds can act as an AT2 receptor antagonist. Fig. 2C also shows the level of IL-6 mRNA production for fibroblasts that were pre-incubated with UNV (10 μM), followed by incubation with C21 (1 μM) and $\text{TNF}\alpha$. The presence of UNV increased the level of IL-6 mRNA production from the level observed with C21 incubation alone, suggesting that UNV may have AT2 agonist properties also (compare " $\text{TNF}\alpha + \text{C21}$ " to " $\text{TNF}\alpha + \text{C21} + \text{UNV}$ ").

32. In this respect, a structural change to the compounds of Allen *et al* to arrive at the presently-claimed compounds has resulted in a completely different (i.e. a **change** in) biological activity, changing an antagonist to an agonist. Moreover, the presently-claimed compounds are AT2 subtype specific, whereas Allen *et al*'s Compound 51 appears to act as an antagonist mainly via AT1, but also possibly via AT2. For compounds that affect AngII signalling, receptor subtype specificity is important so that the desired results (i.e., treating the consequences of hypertension such as inflammation) are targeted. As a direct consequence of this change in activity and receptor subtype specificity, the presently claimed compounds are likely, in my opinion, to have beneficial and superior properties in the clinical setting, for the reasons set out above. In view of this change in biological activity therefore, the structural difference between the compounds of Allen *et al* and the presently claimed compounds do give rise to unexpected, beneficial and superior results.
33. The noted difference in biological activity, however, prevents a further side-by-side showing of unexpected results of the instantly claimed compounds in comparison to those disclosed by Allen *et al*. Such comparisons would require clinical trials which would not possible for ethical reasons (i.e. purely for reasons to obtain a patent).
34. In my opinion, one of ordinary skill in the art seeking to develop AT2 receptor agonists for treating the **consequences** of hypertension, such as inflammation, hypertrophy and fibrosis would not have even looked to Allen *et al*'s teaching regarding AT1 receptor antagonists, much less have been motivated to modify the disclosed AT1 receptor antagonist to arrive at the claimed AT2 receptor agonist compounds. Thus, I believe that it would not have been obvious from a fair reading of Allen *et al*, as a whole, to make the suggested structural modification to produce the claimed AT2 receptor agonist compounds, nor would one of ordinary skill in the art have had a reasonable expectation that the modification would succeed at producing the claimed AT2 receptor agonist compounds.
35. It is for these reasons that I consider the claimed subject matter to be non-obvious over Allen *et al*.

36. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further, that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Date:

11/03/09

Day Month Year


Signed: Thomas Unger

Place: Berlin, Germany

Attachments

Exhibits A to K

Figs. 1A, 1B, 1C, 2A, 2B and 2C

Fig. 1A

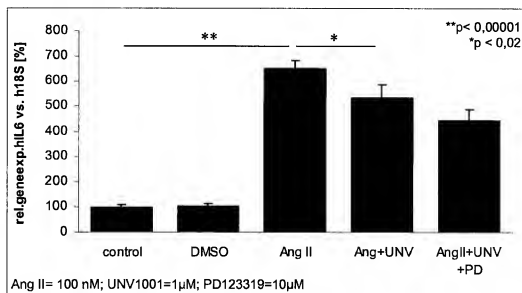


Fig. 1B

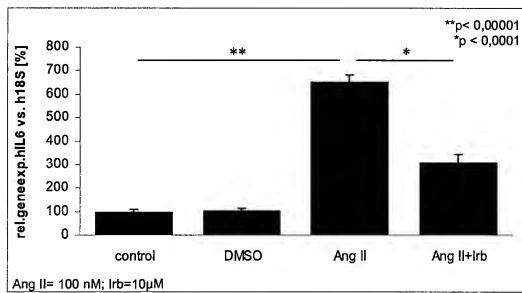


Fig. 1C

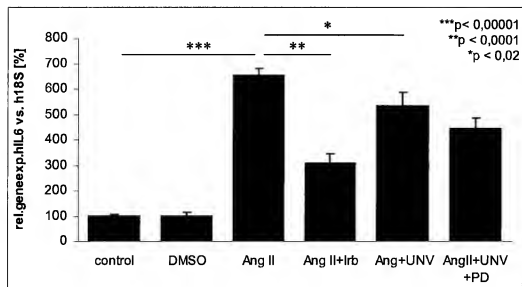


Fig. 2A

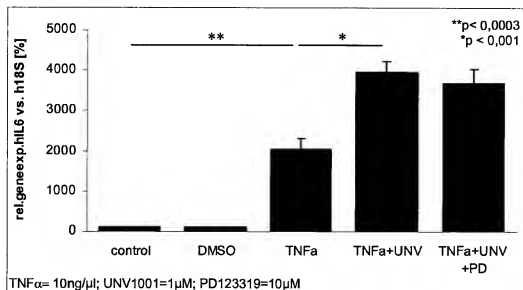


Fig. 2B

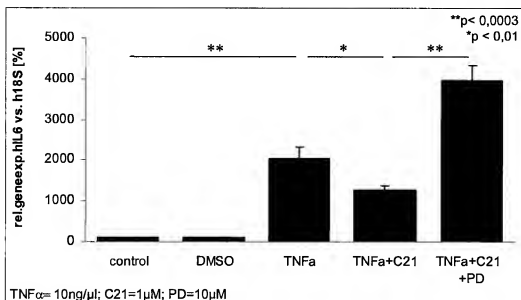
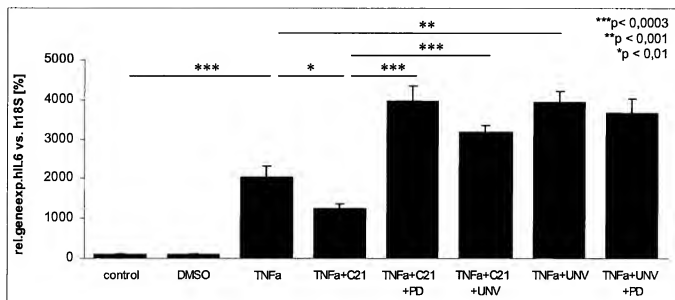


Fig. 2C



**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT A

Publikationsliste Prof. Dr. med. Thomas Unger 1995 -

1995

1. Lebrun C, Blume A, Herdegen Th, Seifert K, Bravo R, **Unger Th** (1995)
Angiotensin II induces a complex activation of transcription factors in the rat brain: Expression of Fos, Jun and Krox proteins.
Neuroscience 65: 93-99
2. Chung O, **Unger Th** (1995)
Role of muscarinic receptors in cardiovascular regulation in SHR.
Progress Hypertension 3: 127-137
3. Edling O, Gohlke P, Paul M, Rettig R, Stauss H, Steckelings UM, **Unger Th** (1995)
The renin angiotensin system (RAS): Physiology and pathophysiology.
In: **ACE inhibitors: Current use and future prospects**. Schachter M (ed) 3-41
4. **Unger Th**, Stoll M (1995)
Effects of AT₁ versus AT₂ receptor stimulation on cell proliferation: physiological and pharmacological implications.
In: **Receptors in Cardiovascular Disease**. Koblika B et al. (ed) 6-10
5. Engelhardt K, Herzog S, **Unger T**, Ziegler A (1995)
Ergebnisse aus den „Disputationes pharmacologicae“ mit der Kieler Ärzteschaft. :
Antiarrhythmika.
Internist prax 35: 149-157
6. Culman J, Höhle S, Qadri F, Edling O, Blume A, Lebrun C, **Unger Th** (1995)
Angiotensin as neuromodulator/neurotransmitter in central control of body fluid and electrolyte homeostasis.
Clin Exp Hypertension 17: 281-293
7. Falkenhahn M, Franke F, Bohle RM, Zhu Yi-Chun, Stauß H, Bachmann S, Danilov S, **Unger Th** (1995)
Cellular distribution of angiotensin converting enzyme after myocardial infarction.
Hypertension 25: 219-226
8. Stauss H, **Unger Th** (1995)
Herzinfarkt und ACE-Hemmer.
ZFA 71: 108-114
9. Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R, **Unger Th** (1995)
The Angiotensin AT₂-receptor mediates inhibition of cell proliferation in coronary endothelial cells.
J Clin Invest 95: 651-657
10. **Unger Th** (1995)
Patient-doctor interactions in hypertension.
J Hum Hypertension 9: 41-45

11. Culman J, Wiegand B, Spitznagel H, Klee S, **Unger Th** (1995)
Effects of the tachykinin NK₁ receptor antagonist, RP 67580, on central cardiovascular and behavioural effects of substance P, neurokinin A and neurokinin B
Br J Pharmacol 114: 1310-1316
12. Höhle S, Spitznagel H, Rascher W, Culman J, **Unger Th** (1995)
Angiotensin AT₁ receptor-mediated vasopressin release and drinking are potentiated by an AT₂ receptor antagonist.
Eur J Pharmacol 275:277-282
13. Linz W, Wiemer G, Gohlke P, **Unger Th**, Schölkens BA (1995)
Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors.
Pharmacological Rev 47:25-49
14. Linz W, Gohlke P, **Unger Th**, Schölkens BA (1995)
Experimental evidence for effects of ramipril on cardiac and vascular hypertrophy beyond blood pressure reduction.
Arch Mal Coeur 88: 31-34
15. Mignat C, **Unger Th** (1995)
ACE inhibitors. Drug interactions of clinical significance.
Drug Safety 12: 334-347
16. Linz W, Wiemer G, Schaper J, Zimmermann R, Nagasawa K, Gohlke P, **Unger Th**, Schölkens BA (1995)
Angiotensin converting enzyme inhibitors, left ventricular hypertrophy and fibrosis.
Mol Cell Biochem 147: 89-97
17. Lebrun C, Bao G, Paul M, Culman J, Möllenhoff E, **Unger Th** (1995)
Angiotensin II induces the expression of c-fos mRNA in the central nervous system of the rat.
Clin Exp Hypertension 17: 877-893
18. Rohmeiss P, Beyer C, Nagy E, Tschöpe C, Höhle S, Strauch M, **Unger Th** (1995)
NaCl injections in brain induce natriuresis and blood pressure responses sensitive to ANG II AT₁ receptors..
Am J Physiol 269: F282-F288
19. Tschöpe C, Jost N, **Unger Th**, Culman J, (1995)
Central cardiovascular and behavioral effects of c- and n-terminal fragments of SP in conscious rats.
Brain Res 690:15-24
20. Höhle S, Blume A, Lebrun C, Culman J, **Unger, Th.** (1995)
Angiotensin receptors in the brain
Pharmacol & Toxicol 77:306-315
21. Gohlke P, **Unger Th** (1995)
Chronic low dose treatment with perindopril improves cardiac function in stroke prone spontaneously hypertensive rats by potentiation of endogenous bradykinin.
Am J Cardiol 76:1E-6E
22. Zhu Yi-Chun, Stauss HM, Bao G, Gohlke P, Zhu Yi-Zhun, **Unger Th** (1995)
Role of bradykinin in the antihypertensive and cardioprotective actions of converting enzyme inhibitors.
Can J Physiol Pharmacol 73: 827-831
23. Culman J, **Unger Th** (1995)
Central tachykinins: mediators of defence reaction and stress reactions.
Can J Physiol Pharmacol 73: 885-891

24. Chung O, Stoll M, **Unger Th** (1995)
Mögliche therapeutische Vorteile einer selektiven Blockade des Angiotensin II AT₁-Rezeptors.
Der Kassenarzt 46: 45-50
25. Zhu Yi-Chun, Stauss HM, Redlich Th, Adamiak D, Mott A, **Unger Th** (1995)
Mechanisms involved in the reduction of infarct size by ACE inhibitor after left coronary artery ligation in rats.
Chin Med J 108 (3): 238
26. Edling O, Bao G, Feelisch M, **Unger Th**, Gohlke P (1995)
Moexipril, a new tissue-specific ACE inhibitor: pharmacological characterization and comparison with enalapril.
J Pharmacol Exp Ther 275: 854-863
27. Rohmeiss P, Beyer C, Quadri F, **Unger Th** (1995)
Osmotically induced natriuresis and blood pressure response involves angiotensin II AT-1 receptors in the subfornical organ.
J Hypertension 13: 1399-1404
28. Stoll M, Meffert S, Stroth U, **Unger Th** (1995)
Growth or anti-growth: angiotensin and the endothelium.
J Hypertension 13: 1529-1534
29. Culman J, Itoi K, **Unger Th** (1995)
Hypothalamic tachykinins: Mediators of stress responses?
Annals of the New York Academy of Sciences 771: 204-218
30. Stauss HM, Mrowka R, Nafz B, Patzak A, **Unger Th**, Persson PB (1995)
Does low frequency power of arterial blood pressure reflect sympathetic tone?
J Auton Nervous Syst 54: 145-154

1996

31. Zhu Yi-Chun, Zhu Yi-Zhun, Spitznagel H, Gohlke P, **Unger Th** (1996)
Substrate metabolism, hormone interaction, and angiotensin-converting enzyme inhibitors in left ventricular hypertrophy.
Diabetes, 45 (Suppl. 1): S59-S65
32. Chung O, **Unger Th**, (1996)
Angiotensin-II-Rezeptor-Antagonisten.
Internist 37: 534-538
33. Höhle S, Culman J, Boser M, Qadri F, **Unger Th** (1996)
Effect of angiotensin AT₂ and muscarinic receptor blockade on osmotically induced vasopressin release.
Eur J Pharmacol 300: 119-123
34. Zhu Yi-Chun, Zhu Yi-Zhun, Stauss HM, **Unger Th** (1996)
ACE inhibitor treatment of congestive heart failure following myocardial infarction.
Cor Europaeum 5: 47-55
35. Adamiak D, Stauss H, Zhu Yi-Chun, Spitznagel H, **Unger Th** (1996)
Die Wirkungen von ACE-Hemmung und ATII-Rezeptorblockade nach Myokardinfarkt bei kinidefizienten Brown-Norway-Katholiek Ratten.
Nieren- und Hochdruckkrankheiten 25: 232-234
36. Chung O, Stoll M, **Unger Th** (1996)
Physiology and Pharmacologic implications of AT₁ versus AT₂ receptors.
Blood Pressure 5: 47-52

37. Spitznagel H, Stauss HM, Zhu Yi-Zhun, Zhu Yi-Chun, Falkenhahn M, **Unger Th** (1996)
Angiotensin II and bradykinin in hypertensive heart disease and myocardial infarction.
High Blood Pressure & Cardiovasc Prevention, 5: 119-123
38. Gohlke P, **Unger Th** (1996)
Ramipril
In: **Cardiovasc Drug Therapy** (ed. F.H. Messerli), W.B. Saunders Company, Philadelphia, London, pp. 755-775
39. Gohlke P, Linz W, Schölkens BA, Wiemer G, **Unger Th** (1996)
Cardiac and vascular effects of long-term losartan treatment in stroke-prone spontaneously hypertensive rats.
Hypertension 28: 397-402
40. Tschöpe C, Gavriluk V, Reinecke A, Seidl U, Riestler U, Hilgenfeldt U, Ritz E, **Unger Th** (1996)
Bradykinin excretion is increased in severely hyperglycemic streptozotocin-diabetic rats.
Immunopharmacology 33: 344-348
41. Meffert S, Stoll M, Steckelings UM, Bottari SP, **Unger Th** (1996)
The angiotensin II AT₂ receptor inhibits proliferation and promotes differentiation in PC12W cells.
Mol Cell Endocrinol 122: 59-67
42. **Unger Th** (1996)
AT₁-Rezeptor-Antagonisten: Eine neue Klasse von Antihypertensiva.
Münch med Wschr 138:754-756
43. Lebrun C, Blume A, Herdegen Th, Möllenhoff E, **Unger Th** (1996)
Complex activation of inducible transcription factors in the brain of normotensive and spontaneously hypertensive rats following central angiotensin II administration.
Regul Pept 66: 19-23
44. Stroth U, **Unger Th** (1996)
Angiotensin-Rezeptor-Antagonisten - die neue Waffe (nicht nur) gegen Bluthochdruck?
Zeitschrift für Allgemeinmedizin 72: 3-6
45. **Unger Th**, Chung O, Csikós T, Culman J, Gallinat S, Gohlke P, Höhle S, Meffert S, Stoll M, Stroth U, Zhu Yi-Zhun (1996)
Angiotensin receptors.
J Hypertension 14 (Suppl 5): S95-S103
46. Gohlke P, Linz W, Schölkens BA, Van Even P, Martorana P, **Unger Th** (1996)
Vascular and cardiac protection by ramipril in spontaneously hypertensive rats: prevention versus regression study.
Br J Clin Practice 84: 1-10
47. **Unger, Th** (1996)
Was leisten AT₁-Rezeptor-Antagonisten?
Therapiewoche 32: 1760-1762

1997

48. Gohlke P, Kuwer I, Schnell A, Amann K, Mall G, **Unger Th** (1997)
Blockade of bradykinin B₂ receptors prevents the increase in capillary density induced by chronic angiotensin-converting enzyme inhibitor treatment in stroke-prone spontaneously hypertensive rats.
Hypertension 29: 478-482

49. Helin K, Völzke H, Tschöpe C, Unger Th, Rettig R (1997)
Die renale Hypertonie.
Fortschritte der Medizin 115: 26-31
50. Unger Th (1997)
Welchen Nutzen bringen die neuen AT₁-Rezeptor-Antagonisten mit sich?
Fortschritte der Medizin 115: 31-33
51. Culman J, Klee S, Ohlendorf C, Unger Th (1997)
Effect of tachykinin receptor inhibition in the brain on cardiovascular and behavioral responses to stress.
J Pharmacol Exp Ther 280:238-246
52. Helin K, Stoll M, Meffert S, Stroth U, Unger Th (1997)
The role of angiotensin receptors in cardiovascular diseases.
Annals of Medicine 29: 23-29
53. Blume A, Lebrun C, Herdegen Th, Linz W, Bravo R, Möllenhoff E, Unger Th (1997)
Increased brain transcription factor expression by angiotensin in genetic hypertension.
Hypertension 29: 592-598
54. Schmitt BM, Unger Th, Rettig R (1997)
Circulating Na⁺/K⁺-ATPase inhibitors: effects of neuropeptides, volume expansion and salt loading in conscious rats.
Clin Exp Pharmacol Physiol 24: 131-138
55. Baulmann J, Unger Th (1997)
Einflüsse des vegetativen- und Zentralnervensystems auf den Bluthochdruck.
In: **Manuale Hypertonologicum, Pathophysiologie des Bluthochdrucks** (ed. Klaus D) Düstri-Verlag Dr. Karl Feistle, Kapitel III-5: pp.1-16
56. Baulmann J, Unger Th (1997)
Endokrine Faktoren.
In: **Manuale Hypertonologicum, Pathophysiologie des Bluthochdrucks** (ed. Klaus D) Düstri-Verlag Dr. Karl Feistle, Kapitel III-6: pp.1-22
57. Csikós T, Gallinat S, Unger Th (1997)
Extrarenal aspects of angiotensin II function.
Eur J Endocrinol, 136: 349-358
58. Tschöpe C, Gohlke P, Zhu Yi-Zhun, Linz W, Schölkens BA, Unger Th (1997)
Antihypertensive and cardioprotective effects after angiotensin-converting enzyme inhibition: role of kinins.
J Cardiac Failure, 3 : 133-147
59. Gallinat S, Csikós T, Meffert S, Herdegen Th, Stoll M, Unger Th (1997)
The angiotensin AT₂ receptor down-regulates neurofilament M in PC12W cells.
Neurosci Lett 227: 29-32
60. Chung O, Unger Th (1997)
Das Renin-Angiotensin-System.
In: **Der Angiotensin II-Antagonismus in der Hypertoniebehandlung: Losartan als Monotherapie oder in Kombination mit Hydrochlorothiazid** (eds. Düsing R, Scholze J, Unger Th) **Klinik & Forschung 3: S5-S7**
61. Chung O, Unger Th (1997)
Selektiver AT₁-Antagonismus.
In: **Der Angiotensin II-Antagonismus in der Hypertoniebehandlung: Losartan als Monotherapie oder in Kombination mit Hydrochlorothiazid** (eds. Düsing R, Scholze J, Unger Th) **Klinik & Forschung 3: S7-S9**

62. Sandmann S, Unger Th (1997)
Blickpunkt Mibefradil.
Aesopus-Verlag, Stuttgart (Blaue Reihe) Unger Th (ed.), pp. 5-63
63. Zhu Yi-Chun, Zhu Yi-Zhun, Gohlke P, Stauss HM, Unger Th (1997)
Effects of angiotensin-converting enzyme inhibition and angiotensin II AT1 receptor antagonism on cardiac parameters in left ventricular hypertrophy.
Am J Cardiol 80: 110A-117A
64. Unger Th, Zhu Yi-Zhun, Stoll M, Gohlke P. (1997)
Basic mechanism in the regulation of myocardial blood flow: angiotensin II and bradykinin.
In: **Coronary microcirculation ischaemia and reperfusion** (eds. Haunso S, Aldershvile J, Svendsen JH) **Alfred Benzon Symposium 41: 104-113**
65. Gallinat S, Edling O, Unger Th (1997)
Das Renin-Angiotensin-System: Physiologie und Pathophysiologie.
In: **AT₁-Rezeptorantagonisten Angiotensin II** (Dominiak P, Unger Th eds.) Steinkopff Verlag, Darmstadt, pp. 1-39
66. Unger Th (1997)
Antihypertensive Therapie: Das Ende der Fahnenstange?
Fortschritte der Medizin 26: 55-57
67. Unger Th (1997)
Neue Antihypertensiva: Anlaß zur Hoffnung.
Fortschritte der Medizin 26: 58-59
68. Unger Th (1997)
Hypertonie: Handlungsbedarf.
Fortschritte der Medizin 28: 25
69. Braun C, Ade M, Unger Th, van der Woude FJ, Gretz N, Rohmeiss P (1997)
Effects of bradykinin and icatibant on renal hemodynamics in conscious spontaneously hypertensive and normotensive rats.
J Cardiovasc Pharmacol 30: 446-454
70. Gohlke P, Tschöpe C, Unger Th (1997)
Bradykinin and cardiac protection.
In: **Hypertension and the Heart** (A. Zanchetti, R.B. Devereux, L. Hansen, S. Goini, eds.) Plenum Publishing Corporation, New York, pp. 159 - pp.172
71. Culman J, Ritter S, Ohlendorf C, Haass M, Maser-Gluth Ch, Spitznagel, H, Unger Th (1997)
A new formalin test allowing simultaneous evaluation of cardiovascular and nociceptive responses.
Can J Physiol and Pharmacol 75: 1203-1211
72. Tschöpe C, Yu M, Reinecke A, Csikós T, Hilgenfeldt U, Pesquero JB, Ritz E, Unger Th (1997)
Renal expression of two rat kallikrein genes under diabetic conditions.
J Hypertension 15: 1711-1714
73. Ito K, Zhu Yi-Zhun, Zhu Yi-Chun, Gohlke P, Unger Th (1997)
Contribution of bradykinin to the cardioprotective action of angiotensin converting enzyme inhibition in hypertension and after myocardial infarction.
Jpn J Pharmacol, 75: 311-318
74. Willenbrock R, Stauss H, Scheuermann M, Osterziel KJ, Unger Th (1997)
Effect of chronic volume overload on baroreflex control of heart rate and sympathetic nerve activity.
Am J Physiol 273: H2580-H2585
75. Lesske J, Fletcher EC, Bao G, Unger Th (1997)
Hypertension caused by chronic intermittent hypoxia - influence of chemoreceptors and

sympathetic nervous system.
J Hypertension 15: 1593-1603

1998

76. Blume A, Seifert K, Lebrun C, Möllenhoff E, Gass P, **Unger Th**, Herdegen Th (1998)
Differential time course of angiotensin-induced AP-1 and Krox proteins in the rat lamina terminalis and hypothalamus.
Neurosci Lett 241: 87-90
77. Maier T, Dai Wen-Jie, Csikós T, Jirikowski GF, **Unger Th**, Culman J. (1998)
Oxytocin pathways mediate the cardiovascular and behavioral responses to substance P in the rat brain.
Hypertension 31: 480-486
78. Steckelings UM, Bottari SP, Stoll M, Wagner T, **Unger Th** (1998)
Repression of c-fos and c-jun gene expression is not part of AT₂ receptor coupled signal transduction.
J Mol Med 76: 202-207
79. Csikós T, Baimforth A, Grojec M, Gohlke P, Culman J, **Unger Th** (1998)
Angiotensin AT₂ degradation is prevented by ligand occupation.
Biochem Biophys Res Commun 243: 142-147
80. Gohlke P, Pees C, **Unger Th** (1998)
AT₂ receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism.
Hypertension 31: 349-355
81. Montgomery HE, Kiernan LA, Whitworth CE, Fleming S, **Unger Th**, Gohlke P, Mullins JJ, McEwan JR. (1998)
Inhibition of tissue angiotensin converting enzyme activity prevents malignant hypertension in TGR (mREN2)27.
J Hypertension 16: 635-643
82. Zhu Yi-Zhun, Zhu Yi-Chun, Gohlke P, **Unger Th** (1998)
Overview on pharmacological properties of angiotensin converting enzyme inhibitors.
In: **ACE Inhibition and Target Organ Protection**, Hall, A.S., Unger, Th. (eds.) Euromed Communications Ltd, U.K. pp. 1-20
83. Chung O, **Unger Th** (1998)
Unopposed stimulation of the angiotensin AT₂ receptor in the kidney.
Nephrol Dial Transplant 13: 537-540
84. Stroth U, Meffert S, Gallinat S, **Unger Th** (1998)
Angiotensin II and NGF differentially influence microtubule proteins in PC12W cells: Role of the AT₂ receptor.
Mol Brain Res 53: 187-195
85. Qadri F, Wolf A, Waldmann Th, Rascher W, **Unger Th** (1998)
Sensitivity of hypothalamic paraventricular nucleus to C- and N-terminal angiotensin fragments: vasopressin release and drinking.
J Neuroendocrinol 10: 275-281
86. Csikós T, Chung O, **Unger Th** (1998)
Receptors and their classification - focus on angiotensin II and the AT₂ receptor.
J Human Hypertension 12: 311-318

87. Unger Th (1998)
Welches Hochdruckmittel? Im Zweifel für die Hochdruckliga.
Selecta 10:11

88. Qadri F, Waldmann Th, Wolf A, Höhle S, Rascher W, Unger Th (1998)
Differential contribution of angiotensinergic and cholinergic receptors in the hypothalamic paraventricular nucleus to osmotically induced AVP release.
J Pharmacol Exp Ther 285: 1012-1018

89. Gallinat S, Yu M, Dorst A, Unger Th, Herdegen Th (1998)
Sciatic nerve transection evokes lasting up-regulation of angiotensin AT₂- and AT₁ receptor mRNA in adult rat dorsal root ganglia and sciatic nerves.
Mol Brain Res 57: 111-122

90. Gallinat S, Unger Th (1998)
Trophic actions mediated by angiotensin AT₂ receptors.
High Blood Press 7: 51-57

91. Sandmann S, Spitznagel H, Chung O, Xia Q-G, Illner S, Jänichen G, Rossius B, Daemen M, Unger Th (1998)
Effects of the calcium channel antagonist Mibefradil on haemodynamic and morphological parameters in myocardial infarction-induced cardiac failure in rats.
Cardiovasc Res 39: 339-350

92. Möllenhoff E, Lebrun C, Blume A, Culman J, Herdegen Th, Unger Th (1998)
Central angiotensin AT₁ and muscarinic receptors in inducible transcription factor expression upon icv NaCl.
Am J Physiol 275: R234-R244

93. Lucius R, Gallinat S, Rosenstiel P, Herdegen Th, Sievers J, Unger Th (1998)
The angiotensin AT₂-receptor promotes axonal regeneration in the optic nerve of adult rats.
J Exp Med 188: 661-670

94. Chung O, Unger Th (1998)
Angiotensin II receptors in the kidney.
Kidney and Blood Pressure Res 21: 245-248

95. Chung O, Köhl H, Stoll M, Unger Th (1998)
Physiological and pharmacological implications of AT₁ versus AT₂ receptors.
Kidney Int 54: S95-S99

96. Stoll M, Unger Th (1998)
The renin angiotensin system, the endothelium and vascular diseases.
In: **Modern Visualisation of Endothelium**, Polak, J, (ed.) Harwood Academic Publisher, Reading, UK, 113-132

97. Unger Th (1998)
Zusammenfassung der Arbeitsgruppe 1.
In: **Zwei Jahrhunderte Wissenschaft und Forschung in Deutschland. Entwicklungen – Perspektiven**, v. Engelhardt D, (ed.) Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 214-215

98. Fischer J, Tschöpe C, Reinecke A, Giachelli CM, Unger Th (1998)
Upregulation of osteopontin expression in renal cortex of streptozotocin-induced diabetic rats is mediated by bradykinin.
Diabetes 47: 1512-1518

99. Nurminen M-L, Culman J, Haass M, Chung O, Unger Th (1998)
Effect of moxonidine on blood pressure and sympathetic tone in conscious spontaneously

hypertensive rats.
Eur J Pharmacol 362: 61-67

100. **Unger Th**, (1998)
Angiotensin-AT₁ Rezeptorantagonisten: Pharmakologisches Profil und klinisches Profil.
NBP-Informationen (Hochdruckliga) 4: 2-3
101. Gallinat S, Gohlke P, **Unger Th** (1998)
AT₂-Rezeptoren: Zelldifferenzierung, Gewebsregeneration und Apoptose.
Pressure, Newsletter, Springer-Verlag, Heidelberg 2:4
102. Chung O, **Unger Th** (1998)
Pharmacology of angiotensin receptors and AT₁ receptor blockers.
Basic Res Cardiof 93: 15-23
103. **Unger Th**, Culman J, Gohlke P (1998)
Angiotensin II receptor blockade and end-organ protection: pharmacological rationale and evidence.
J. Hypertension 16: S3-S9
104. Chung O, Csikós T, **Unger Th** (1998)
Pharmacologic differences of angiotensin II antagonists.
Receptors in Cardiovascular Disease 5: 1-6
105. Csikós T, **Unger Th** (1998)
Angiotensin II receptors.
High Blood Press 7: 97-101
106. Conlín PR, Chung O, Gansevoort RT, London GM, **Unger Th**, Weir MR (1998)
Papel de la angiotensina II en la enfermedad cardiovascular y renal.
In: **Antagonistas De Los Receptores De La Angiotensina II Y Protección renal**.
Ruilope, LM (ed.) Sociedad Española de Hipertensión, pp. 38 – 51
107. **Unger Th** (1998)
Calciumantagonisten und AT₁-Rezeptorantagonisten - pharmakologische Fortschritte.
Proceedings of the Symposium Lacidipin/Telmisartan, (Tagung der Hochdruckliga, Freiburg) pp. 6-11
- 1999**
108. Gallinat S, Busche S, Schütze St, Krönke M, **Unger Th** (1999)
AT₂-receptor stimulation induces generation of ceramides in PC12W cells.
FEBS Lett 443: 75-79
109. Blume A, Herdegen T, **Unger Th** (1999)
Angiotensin peptides and inducible transcription factors.
J Mol Med 77: 339-357
110. Csikós T, Chung O, **Unger Th** (1999)
Role of angiotensin II in hypertension-induced cardiac hypertrophy and failure.
Heart Failure Reviews 3: 159-168
111. Chung O, Csikós T, **Unger Th** (1999)
Angiotensin II receptor pharmacology and AT₁-receptor blockers
J Human Hypertension 13 (Suppl 1): S11-S20
112. Culman J, von Heyer Ch, Piepenburg B, Rascher W, **Unger Th** (1999)
Effects of systemic treatment with irbesartan and losartan on central responses to angiotensin II in conscious, normotensive rats.
Eur J Pharmacol 367: 255-265

113. Stroth U, Unger Th (1999)
The renin-angiotensin-system and its receptors.
J Cardiovasc Pharmacol 33 (Suppl 1): S21-S28
114. Unger Th (1999)
Welchen praktischen Stellenwert haben moderne Thiaziddiuretika?
Fortschritte der Medizin 117: 14-15
115. Chung O, Unger Th (1999)
Pharmakologische Aspekte von Angiotensin II-Rezeptoren und AT₁-Rezeptorantagonisten.
In: **AT₁-Rezeptorblockade**. Dominiak P, Heusch G (eds.) Steinkopff Verlag Darmstadt, pp. 25-37
116. Gohlke P, Jürgensen T, von Kügelgen S, Unger Th (1999)
Candesartan cilexetil: development and preclinical studies.
Drugs of Today 35 (2): 105-115
117. Reinecke A, Unger Th (1999)
Blickpunkt Irbesartan.
Aesopus-Verlag, Stuttgart (Blaue Reihe) pp 1-85
118. Gallinat S, Edling O, Unger Th (1999)
Das Renin-Angiotensin-System: Physiologie und Pathophysiologie.
In: **AT₁-Rezeptorantagonisten Angiotensin II**. Dominiak P, Unger Th (eds). Steinkopff Verlag, Darmstadt, pp. 1-38
119. Chung O, Unger Th (1999)
Angiotensin-II-AT₁-Rezeptorantagonisten.
Kliniker 8/28: 248-252
120. Sandmann S, Min J-Y, Meissner A, Unger Th (1999)
Effects of the calcium channel antagonist mibefradil on haemodynamic parameters and myocardial Ca²⁺-handling in infarct-induced heart failure in rats.
Cardiovasc Res 44: 67-80
121. Dai WJ, Funk A, Culman J, Herdeggen Th, Unger Th (1999)
Blockade of central angiotensin AT₁ receptors improves neurological outcome and reduces expression of AP-1 transcription factors after focal brain ischemia in rats.
Stroke 30: 2391-2399
122. Unger Th (1999)
Björn Folkow Award Lecture.
The angiotensin AT₂ receptor: Variations on an enigmatic theme.
J Hypertension 17: 1775-1786
123. Sandmann S, Unger Th (1999)
L- and T-type calcium channel blockade - the efficacy of the calcium channel antagonist mibefradil.
J Clin Basic Cardiol 2: 187-201
124. Unger Th (1999)
Significance of angiotensin type 1 receptor blockade: Why are angiotensin II receptor blockers different?
Am J Cardiol 84: 9S-15S
125. Zhu Yi-Chun, Falkenhahn M, Franke F, Bohle RM, Stauss HM, Danilov S, Unger Th (1999)
Expression of cardiac angiotensin-converting enzyme after myocardial infarction.
Acta Pharmacol Sin 20 (2): 97-102
126. Tschöpe C, Koch M, Spillmann F, Heringer-Walther S, Mochmann H-C, Stauss H, Bader M, Unger Th, Schultheiss H-P, Walther Th (1999)

Upregulation of cardiac bradykinin B2 receptors after myocardial infarction.
Immunopharmacology 44: 111-117

127. Min JY, Sandmann S, Meissner A, **Unger Th**, Simon R (1999)
Differential effects of mibefradil, verapamil, and amlodipine on myocardial function and intracellular Ca^{2+} handling in rats with chronic myocardial infarction.
J Pharmacol Exp Ther 29 (3): 1038-1044
 128. Xia Q-G, Chung O, Spitznagel H, Sandmann S, Illner S, Rossius B, Jähnichen G, Reinecke A, Gohlke P, **Unger Th** (1999)
Effects of a novel angiotensin AT₁ receptor antagonist, HR720, on rats with myocardial infarction.
Eur J Pharmacol 385: 171-179
 129. Lucius R, Gallinat S, Busche S, Rosenstiel P, **Unger Th** (1999)
Beyond the blood pressure - new roles for angiotensin II.
Cell Mol Life Sci 56: 1008-1019
 130. Tschöpe C, Reinecke A, Seidl U, Yu M, Gavriluk V, Riester U, Gohlke P, Graf K, Bader M, Hilgenfeldt U, Pesquero JB, Ritz E, **Unger Th** (1999)
Functional, biochemical, and molecular investigations of the renal kallikrein-kinin system in diabetic rats.
Am J Physiol 277: H2333-H2340
 131. Tschöpe C, Walther Th, Yu M, Reinecke A, Pesquero JB, Bader M, Schultheiss HP, **Unger Th** (1999)
Myocardial expression of rat bradykinin receptors and two tissue kallikrein genes in experimental diabetes.
Immunopharmacology 44: 35-42
 132. Chung O, **Unger Th** (1999)
Angiotensin II receptor blockade and end-organ protection.
Am J Hypertens 12: S150-S156
 133. Zhu Yi-Chun, Zhu Yi-Zhun, Li J, Schäfer H, Schmidt WE, Yao T, **Unger Th** (1999)
Effects of ramipril on cardiac gene transcription levels of angiotensin II receptors after myocardial infarction.
Acta Pharmacol Sin 20 (6): 481-485
- 2000**
134. **Unger Th** (2000)
Neurohormonal modulation in cardiovascular disease.
Am Heart J 139: I, Part 2, S2-S8
 135. Baulmann J, Spitznagel H, Herdegen T, **Unger Th**, Culman J (2000)
Tachykinin receptor inhibition and c-Fos expression in the rat brain following formalin-induced pain.
Neuroscience 95: 813-820
 136. Chung, O, Hinder M, Sharma A, Bönner G, Middeke M, Platon J, **Unger Th** (2000)
Comparison on the efficacy and safety of losartan (50-100 mg) with the T-type calcium channel blocker mibefradil (50-100 mg) in mild to moderate hypertension.
Fundam Clin Pharmacol 14: 31-41
 137. Spitznagel H, Chung O, Xia Q-G, Rossius B, Illner S, Jähnichen G, Sandmann, S, Daemen MJAP, **Unger Th** (2000)
Cardioprotective effects of the Na^+/H^+ -exchange inhibitor cariporide in infarct-induced heart failure in rats.
Cardiovasc Res 46:102-110

138. Zhu Yi-Zhun, Zhu Yi-Chun, Stoll M, **Unger Th** (2000)
Identification of eight up-regulated genes in rat heart after myocardial infarction by means of differential mRNA display.
Jpn Heart J 41: 59-66
139. Sandmann S, Bohle RM, Dreyer T, **Unger Th** (2000)
The T-type calcium channel blocker mibefradil reduced interstitial and perivascular fibrosis and improved haemodynamic parameters in myocardial infarction-induced cardiac failure in rats.
Virchows Arch 436: 147-157
140. **Unger Th** (2000)
Differences among angiotensin II type 1 receptor blockers: characteristics of candesartan cilexetil.
Blood Pressure 9 (Suppl 1): 14-18
141. Chung O, **Unger Th** (2000)
Mechanisms of action of angiotensin II receptor antagonists and differences from other drugs acting on the renin-angiotensin system.
In: **Angiotensin II Receptor Antagonists in perspective** (Mancia G, ed.), Dunitz M, London, pp 1-19
142. Busche S, Gallinat S, Bohle RM, Reinecke A, Seebeck J, Franke F, Fink L, Zhu M, Sumners C, **Unger Th** (2000)
Expression of angiotensin AT1- and AT2 receptors in adult rat cardiomyocytes after myocardial infarction: A single-cell RT-PCR study.
Am J Pathology 157:605-611
143. Stroth U, Blume A, Mielke K, **Unger Th** (2000)
Angiotensin AT₂ receptor stimulates ERK1 and ERK2 in quiescent but inhibits ERK in NGF-stimulated PC12W cells.
Mol Brain Res 78: 175-180
144. de Gasparo M, Catt KJ, Inagami T, Wright JW, **Unger Th** (2000)
The angiotensin II receptors.
Pharmacol Rev 52 (3): 415-472
145. Clausmeyer S, Reinecke A, Stürzebecher, R, **Unger Th**, Peters J (2000)
Tissue-specific expression of a rat renin transcript lacking the coding sequence for the prefragment and its stimulation by myocardial infarction.
Endocrinology 141: 2963-2970
146. Zhu Y-Z, Zhu Y-C, Li J, Schäfer H, Schmidt W, Yao T, **Unger Th** (2000)
Effects of losartan on hemodynamic parameters- and angiotensin receptor mRNA levels of rat heart after myocardial infarction.
JRAAS 1: 257-262
147. Hoheisel U, **Unger Th**, Mense S (2000)
A block of spinal nitric oxide synthesis leads to increased background activity predominantly in nociceptive dorsal horn neurones in the rat.
Pain 88: 249-257
148. **Unger Th**, Azizi M, Belz GG (2000)
Blocking the tissue renin-angiotensin system:the future cornerstone of therapy.
J Human Hypertension 14: S23 - S31
149. **Unger Th** (2000)
Hypertonie 2000
Dtsch med Wschr 125:1383

150. **Unger Th, Sandmann, S (2000)**
Angiotensin receptor blocker selectivity at the AT₁- and AT₂-receptors: conceptual and clinical effects.
JRAAS 1: 56-59
- 2001**
151. **Unger Th (2001)**
Inhibiting renin-angiotensin in the brain: the possible therapeutic implications.
Blood Pressure 10 (suppl 1) 12-16
152. Xia Q-G, Chung O, Spitznagel H, Illner S, Jänichen G, Rossius B, Gohlke P, Reinecke A, **Unger Th (2001)**
Significance of timing of angiotensin AT₁ receptor blockade in rats with myocardial infarction-induced heart failure.
Cardiovasc Res 49: 110-117
153. Sandmann S, Claas R, Cleutjens JPM, Daemen MJAP, **Unger Th (2001)**
Calcium channel blockade limits cardiac remodeling and improves cardiac function in myocardial infarction-induced heart failure in rats.
J Cardiovasc Pharmacol 37: 64-77
154. Sandmann S, Yu M, **Unger Th (2001)**
Transcriptional and translational regulation of calpain in the rat heart after myocardial infarction. - Effects of AT₁ and AT₂ receptor antagonists and ACE inhibitor.
Brit J Pharmacol 132 : 767-777
155. Blume A, Kaschina E, **Unger Th (2001)**
AT₂ receptors: Signalling and pathophysiologic role.
Curr Opin Nephrol Hypertens 10: 239-246
156. **Unger Th (2001)**
The cardiovascular continuum - Pathophysiology and Targets for Therapy.
In: **Prevention of Disease Progression Throughout the Cardiovascular Continuum** (Ryden, L.E, ed.), **Springer-Verlag Berlin, pp 2 – 15**
157. Möllenhoff E, Herdegen Th, Lebrun C, Chatterjee B, Blume A, **Unger Th (2001)**
Effect of repetitive iv injections of ANG II on c-Fos and AT₁ receptor expression in the rat brain.
Am J Physiol 280: R1095-R1104
158. **Unger Th (2001)**
Hypertonie 2000 (Leserbriefe).
Dtsch med Wschr 126: 384
159. Stoll M, **Unger Th (2001)**
Angiotensin and its AT₂ receptor: new insights into an old system.
Regul Pept 99 (2-3): 175-182
160. Sandmann S, Yu Minghuan, Kaschina E, Blume A, Bouzinova E, Aalkjaer Ch, **Unger Th (2001)**
Differential effects of angiotensin AT₁ and AT₂ receptors on the expression, translation and function of the Na⁺-H⁺ exchanger and Na⁺-HCO₃ cotransporter in the rat heart after myocardial infarction.
J Am Coll Cardiol 37: 2154-2165
161. Culman J, Baulmann J, Blume A, **Unger Th (2001)**
The renin-angiotensin system in the brain: an update.
JRAAS 2: 96-102

162. Gohlke P, Weiss S, Jansen A, Wiene W, Stangier J, Rascher W, Culman J, **Unger Th** (2001)
The AT₁ receptor antagonist, telmisartan, administered peripherally inhibits central responses to angiotensin II in conscious rats.
J Pharmacol Exp Ther 298: 62-70
 163. Zhu YZ, Zhu YC, Wang ZJ, Lu Q, Lee HS, **Unger Th** (2001)
Time-dependent apoptotic development and pro-apoptotic genes expression in rat heart after myocardial infarction.
Jpn J Pharmacol 86(3): 355-358
 164. **Unger Th** (2001)
Pharmacology of AT₁-receptor blockers.
Blood Pressure 10: (Suppl. 3) 5-10
 165. Fischer J, Stoll M, Hahn AWA, **Unger Th** (2001)
Differential regulation of Thrombospondin-1 and Fibronectin expression by angiotensin II receptor subtypes in cultured endothelial cells.
Cardiovasc Res 51 (4): 784-791
 166. Spitznagel H, Baulmann J, Blume A, Herdegen Th, **Unger Th**, Culman J (2001)
C-Fos expression in the rat brain in response to substance P and neurokinin B.
Brain Res 915 11-21
 167. Blüm T, Hoheisel U, Unger Th, Mense S (2001)
Fibroblast growth factor-2 acutely influences the impulsive activity of rat dorsal horn neurones.
Neurosci Research 40 115-123
 168. **Unger Th** (2001)
Pharmacological properties of angiotensin II antagonists: examining all the therapeutic implications
JRAAS 2, Supplement 2: 4 – 7
 169. Dzau VJ, Bernstein K, Celermajer D, Cohen J, Dahlöf B, Deanfield J, Diez J, Drexler H, Ferrari R, van Gilst W, Hansson L, Hornig B, Husain A, Johnston C, Lazar H, Lonn E, Luscher T, Mancini J, Mimran A, Pepine C, Rabelink T, Remme W, Ruijter L, Ruzicka M, Schunkert H, Swedberg K, **Unger Th**, Vaughan D, Weber M. (2001)
The relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and endpoint data.
Am J Cardiol 88(suppl):1L-20L
- 2002**
170. Dzau VJ, Bernstein K, Celermajer D, Cohen J, Dahlöf B, Deanfield J, Diez J, Drexler H, Ferrari R, van Gilst W, Hansson L, Hornig B, Husain A, Johnston C, Lazar H, Lonn E, Luscher T, Mancini J, Mimran A, Pepine C, Rabelink T, Remme W, Ruijter L, Ruzicka M, Schunkert H, Swedberg K, **Unger Th**, Vaughan D, Weber M (2002)
Working group on tissue angiotensin converting enzyme, international society of cardiovascular pharmacotherapy
Cardiovasc Drugs & Ther 16:149-160
 171. Sandmann S, Seebeck J, **Unger Th** (2002)
Bedeutung und Regulation spannungsabhängiger Kalziumkanäle.
In: **Handbuch der Kalziumkanalblocker** (Bönner G, Dominiak P, eds.) Schattauer-Verlag, Stuttgart, S. 63-86
 172. **Unger Th** (2002)
The Role of the Renin-Angiotensin System in the development of Cardiovascular Disease.
Am J Cardiol. 2002; 89 (suppl): 3A-10A

173. Stoll M, Hahn AW, Jonas U, Zhao Y, Schieffer B, Fischer JW, **Unger Th** (2002)
Identification of a Zinc Finger Homeodomain Enhancer Protein After AT₂ Receptor
Stimulation by Differential mRNA Display.
Arterioscler Thromb Vasc Biol 22(2):231-7
174. Blume A, Neumann C, Dorenkamp M, Culman J, **Unger Th** (2002)
Involvement of adrenoceptors in the angiotensin II-induced expression of Inducible
transcription factors in the rat forebrain and hypothalamus.
Neuropharmacology 42(2):281-8
175. Rosenstiel P, Gallinat S, Artl A, **Unger Th**, Sievers J, Lucius R (2002)
Angiotensin AT₂ receptor ligands- do they have a potential as future treatments for
neurological disease?
Current Opinion CNS Drugs 16(3):145-153
176. Kaschina E, **Unger Th** (2002)
Effets des Antagonistes des Récepteurs AT₁ sur l'Endothélium Vasculaire.
In: **Actualité Néphrologie de l'hôpital necker**, Éditions Flammarion, Paris, pp 31-50
177. Stoll M, Kwitek-Black A, Jacob H J, **Unger Th** (2002)
The Role of Genetics in the Pathology of Hypertension.
in: **New Frontiers in the Management of Hypertension** (eds.: N. Kaplan, M. Schachter),
Lippincott Williams & Wilkins, London, pp 17-31
178. Sandmann S, Prenzel F, Shaw L, Schauer R, **Unger Th** (2002)
Activity profile of calpain I and II in chronically infarcted rat myocardium - influence of the
calpain inhibitor CAL 9561.
Brit J Pharmacol 135(8):1951-1958
179. Scheifer C, Hoheisel U, Trudung P, **Unger Th**, Mense S (2002)
Rats with chronic spinal cord transection as a possible model for the AT-level pain of
paraplegic patients.
Neurosci Lett 323:117-120
180. Sandmann S, **Unger Th** (2002)
Implications physiopathologiques et cliniques des récepteurs AT₁/AT₂ de l'angiotensine II
dans l'insuffisance cardiaque, coronaire et rénale
Drugs 62(1): 43-52
181. **Ungert Th** (2002)
Was leisten ACE-Hemmer und Antagonisten jenseits der Blutdrucksenkung?
MedReport 26(20): 2
182. Gohlke P, Kox T, Jürgensen T, von Kögelgen S, Rascher W, **Unger Th**, Culmann J (2002)
Peripherally applied candesartan inhibits central responses to angiotensin II in conscious rats
Naunyn-Schmiedeberg's Arch Pharmacol 365: 477-483
183. **Unger, Th** (2002) Laudatio
Herz 27 (Suppl. I) 6-7
184. Culman J, Blume A, Gohlke P, **Unger Th** (2002)
The renin-angiotensin system in the brain: possible therapeutic implications for AT₁ –receptor
blockers
J Human Hypertens 16: S64-S70
185. Gohlke P, Jürgensen T, Kox T, von Kögelgen S, Rascher W, Culman J, **Unger Th** (2002)
Effects of peripherally applied candesartan and candesartan cilexetil on central responses to
angiotensin II in conscious rats.
J Hypertension 20(5): 909-18

186. **Unger Th**, Rangoonwala B, Rosenthal J (2002)
Differentialtherapie mit ACE-Hemmern bzw. AT1-Blockern
MMW-Fortschritte der Medizin 24:36-38
187. Sandmann S, Spormann J, Prenzel F, Shaw L, **Unger Th** (2002)
Calcium Channel Blockade limits Transcriptional, Translational and Functional Up-regulation of the Cardiac Calpain System after Myocardial Infarction.
Europ J Pharmacol 453: 99-109
188. Sandmann S, **Unger Th** (2002)
Pathophysiological and clinical implications of AT1 and AT2 receptors in heart failure and coronary and renal failure.
Drugs 62 (Spec Iss 1): 43-52
189. **Unger Th** (2002)
Neue Optionen in der medikamentösen Hochdruckbehandlung: Anspruch und Wirklichkeit.
DMW Dtsch Med Wochenschr 127: 2404-2406
- 2003**
190. Sandmann S, Kashina E, Blume A, Kruse M-L, **Unger Th** (2003)
Bradykinin B₁ and B₂ receptors differentially regulate cardiac Na⁺-H⁺ exchanger, Na⁺-Ca²⁺ exchanger and Na⁺-HCO₃⁻ symporter
Eur J Pharmacol 458:3-16
191. Reinöhl J, Hoheisel U, **Unger Th**, Mense S (2003)
Adenosine triphosphate as a stimulant for nociceptive and non-nociceptive muscle group IV receptors in the rat
Neurosci Lett 338:25-28
192. Zhao Y, Biermann T, Luther C, **Unger Th**, Culman J, Gohlke P (2003)
Contribution of bradykinin and nitric oxide to AT2 receptor-mediated differentiation in PC12 W cells
J Neurochem;85(3):759-67
193. Pees Ch, **Unger Th**, Gohlke P (2003)
Effect of angiotensin AT2 receptor stimulation on vascular cyclic GMP production in normotensive Wistar Kyoto rats
Int J Biochem Cell Biol 35:963-972
194. Tschöpe C, Seidl U, Reinecke A, Riester U, Graf K, Schultheiss H P, Hilgenfeldt U, **Unger Th** (2003)
Kinins are involved in the antiproteinuric effect of angiotensin-converting enzyme inhibition in experimental diabetic nephropathy
Int immunopharmacol 3:335-344
195. Hoheisel U, Scheifer C, Trundrup P, **Unger Th**, Mense S (2003)
Pathophysiological activity in rat dorsal horn neurones in segments rostral to a chronic spinal cord injury.
Brain Res 974:134-145
196. Kintscher U, Bruemmer D, Blaschke F, **Unger Th**, Law RE (2003)
p38 MAP kinase negatively regulates angiotensin II-mediated effects on cell cycle molecules in human coronary smooth muscle cells.
Biochem Biophys Res Comm 305:552-556

197. Lottemoser K, **Unger Th**, Gohlke P, Vetter H, Duesing R (2003)
Differential Effect of Acute Angiotensin II Type 1 Receptor Blockade on the Vascular and Adrenal Response to Exogenous Angiotensin II in Humans.
Am J Hypertens 16:445-452
198. **Unger Th**, Kaschina E (2003)
Drug interactions with angiotensin receptor blockers: a comparison with other antihypertensives.
Drug Saf 26:707-720
199. Kaschina E, **Unger Th** (2003)
Angiotensin AT1/AT2 receptors: regulation, signalling and function.
Blood Press 12:70-88
200. Kintscher U, **Unger Th** (2003)
Angiotensin II receptor expression: from maturation to pathogenesis.
Am J Physiol Regul Integr Comp Physiol 285:R26-R27
201. **Unger Th** (2003)
The ongoing telmisartan alone and in combination with ramipril global endpoint trial programm.
Am J Cardiol 91:28G-34G
202. Seebeck J, **Unger Th** (2003)
ACE-Hemmer, AT₁-Rezeptorantagonisten, Diuretika, direkte Vasodilatoren.
in: **Arzneimitteltherapie der Herz-Kreislaufkrankheiten, Teil B, Spezielle Pharmakologie** (Böhm M, Eschenhagen Th. eds.) **Wiss Verlagsgesellschaft, Stuttgart**, 182-191
203. **Unger Th** (2003)
Angiotensin und seine Rezeptoren
Pressure 1/2003 5-6
204. **Unger Th** (2003)
Blood pressure lowering and rennin-angiotensin system blockade.
J Hypertens 21: (suppl 6) S3-S7
205. Regitz-Zagrosek V, **Unger Th** (2003)
Angiotensin-Rezeptorantagonisten - Therapeutischer Einsatz bei Hypertonie und Herzinsuffizienz.
Bremen: **UNI-MED**, 116 S. (UNI-MED SCIENCE)
206. Reinecke K, Lucius R, Reinecke A, Rickert U, Herdegen T, **Unger Th** (2003)
Angiotensin II accelerates functional recovery in the rat sciatic nerve in vivo: role of the AT₂ receptor and the transcription factor NF- κ B.
FASEB J 17:2094-2096, <http://www.fasebj.org/cgi/doi/10.1096/fj.02-1193fje>
207. **Unger Th** (2003)
Inhibiting angiotensin receptors in the brain: possible therapeutic implications.
Curr Med Res Opin 19:449-451
208. Scholze J, **Unger Th** (2003)
Die ALLHAT-Studie. Primäres. Antihypertensivum – Diuretika vs. Kalziumantagonisten vs. ACE-Hemmer.
Internist 44:1193-1195
209. Groth W, Blume A, Gohlke P, **Unger Th**, Culman J (2003)
Chronic pretreatment with candesartan improves recovery from focal cerebral ischaemia in rats.

210. **Unger Th** (2003)
ALLHAT-Studie: Kontra
Berliner Ärzte 40:19-21
211. Wittchen HU, Ritz E, Göke B, Lehnert H, Kirch W, Pittrow D, Krause P, Höfler M, **Unger T**, Bramlage P, Tschöpe D, Sharma A (2003)
Diabetiker mit erhöhtem Blutdruck in der Hausarztpraxis – 38% haben bereits eine Mikroalbuminurie!
MMW Fortschr Med 145:43-44
212. **Unger Th** (2003)
Diuretika – Medikamentöse Therapie der Wahl bei unkomplizierter Hypertonie? -Contra
Dtsch Med Wochenschr 128:2491-2492
213. **Unger Th** (2003)
Laudatio – Auszeichnung für die Erforschung von Acarbose
Medizinische Klinik 98:(Suppl I) 18-19
214. Tiede K, Stöter K, Petrik C, Chen W.-B, Ungefroren H, Kruse M.-L, Stoll M, **Unger Th**, Fischer J W (2003)
Angiotensin II AT₁-receptor induces biglycan in neonatal cardiac fibroblasts via autocrine release of TGF β in vitro.
Cardiovasc Res 60:538-546
215. Friedrich M G, Dahlöf B, Sechem U, **Unger Th**, Knecht M, Dietz R, (2003)
Telmisartan effectiveness on left ventricular mass reduction (TELMAR) as assessed by magnetic resonance imaging in patients with mild-to-moderate hypertension – a prospective, randomised, double-blind comparison of telmisartan with metoprolol over a period of six months – rationale and study design.
J Renin Angiotensin Aldosterone Syst 4:234-243
216. Wittchen HU, Krause P, Höfler M, Pfister H, Ritz E, Göke B, Lehnert H, Tschöpe D, Kirch W, Pittrow D, Bramlage P, Sharma A M, Bramlage P, Kupper B, **Unger T** (2003)
Arterielle Hypertonie, Diabetes mellitus und assoziierte Erkrankungen in der Allgemeinarztpraxis
Fortschr Med Orig 121:(Suppl 1) 19-27
217. Krause P, Wittchen HU, Kupper B, Pittrow D, **Unger T**, Sharma A M, Ritz E, Göke B, Lehnert H, Tschöpe D, Kirch W, Pfister H, Bramlage P, Höfler M (2003)
Wie beurteilen Ärzte Ihre Versorgungspraxis bei Diabetes mellitus und arterieller Hypertonie?
Fortschr Med Orig 121:(Suppl 1) 12-18
218. Wittchen HU, Krause P, Höfler M, Pfister H, Kupper B, Pittrow D, Bramlage P, **Unger T**, Sharma A M, Ritz E, Göke B, Lehnert H, Tschöpe D, Kirch W, (2003)
Ziel, Design und Methodik der „Hypertension and Diabetes Risk Screening and Awareness“- (HYDRA)-Studie
Fortschr Med Orig 121:(Suppl 1) 2-11
219. **Unger T** (2003)
Antihypertensive Therapie. Der Druck zu handeln bleibt
Der Hausarzt 40: 48-52
- 2004
220. Kusserow H, **Unger Th** (2004)
Vasoactive peptides, their receptors and drug development

221. Marshall RP, Gohlke P, Chambers RC, Howell DC, Bottoms S, Unger Th, McNulty RJ, Laurent GJ (2004)
Angiotensin II and the fibroproliferative response to acute lung injury.
Am J Physiol Lung Cell Mol Physiol 286:L156-L164
222. Gallinat S, Edling O, Unger Th (2004)
Pathophysiologie des Renin-Angiotensin Systems
In: **Arteriellen Hypertonie** (J: Rosenthal, R: Kolloch) Heidelberg: Springer , 4. Aufl., S. 225-240
223. Chung O, Unger Th (2004)
Angiotensin AT1-Rezeptor Antagonisten.
In: **Arteriellen Hypertonie** (J: Rosenthal, R: Kolloch) Heidelberg: Springer , 4. Aufl., S. 647-656
224. Sharma AM, Wittchen H-U, Kirch W, Pittrow D, Ritz E, Göke B, Lehnert H, Tschöpe D, Krause P, Höfler M, Pfister H, Bramlage P, Unger Th (2004)
High prevalence and poor control of hypertension in primary care: cross sectional study
J Hypertens 22:479-486
225. Kintscher U, Clasen R, Unger Th (2004)
Verbesserung des Insulin- und Glukosestoffwechsels unter AT1 Blockern
Pressure 1/2004:3
226. Kaschina E, Steckelings M, Unger Th (2004)
Hypertension, Renin and
In: **The Encyclopedia of Endocrinology and Endocrine Diseases. Vol. 2** (L. Martini ed.) Elsevier/Academic Press, San Diego
227. Unger Th, Schölkens B (eds.) (2004)
Handbook of Experimental Pharmacology. Vol. 163/I-II: Angiotensin
Springer, Berlin, 1151 pages
228. Wruck C, Stoll M, Unger Th (2004)
AT₂ Function and Target Genes
In: **Handbook of Experimental Pharmacology. Vol. 163/ I: Angiotensin** (T. Unger, B. Schölkens eds.)
Springer, Berlin, pp 449-475
229. Unger Th (2004)
Preface
In: **Handbook of Experimental Pharmacology. Vol. 163/ I: Angiotensin** (T. Unger, B. Schölkens eds.)
Springer, Berlin, pp V-VI
230. Steckelings UM, Stoppelhaar M, Sharma AM, Wittchen HU, Krause P, Küpper B, Kirch W, Pittrow D, Ritz E, Göke B, Lehnert H, Tschöpe D, Höfler M, Pfister H, Unger Th (2004)
HYDRA: Possible determinants of unsatisfactory hypertension control in german primary care patients
Blood Pressure 13:80-88
231. Schupp M, Janke J, Clasen R, Unger Th, Kintscher U (2004)

Angiotensin Type 1 receptor blockers induce peroxisome proliferator-activated receptor- γ activity
Circulation 109:2054-2057

232. Pittrow D, Kirch W, Bramlage P, Lehnert H, Hoffer M, **Unger Th**, Sharma AM, Wittchen HU (2004)
 Patterns of antihypertensive drug utilization in primary care
Eur J Clin Pharmacol 60:135-142
233. **Unger Th** (2004)
 Lessons from ALLHAT: Are low budget diuretics first line therapy?
Z Kardiol 93:349-356
234. Zimmermann M, **Unger Th** (2004)
 Challenges in improving prognosis and therapy: The ongoing Telmisartan alone and in combination with Ramipril global endpoint trial program
Expert Opin Pharmacother 5:1201-1208
235. Xia QG, Reinecke A, Dorenkamp M, Storz C, Bitterling H, Penz S, Cleutjens J, Daemen MJ, Simon R, **Unger Th** (2004)
 Comparison of cardioprotective effects of mibefradil and ramipril in stroke-prone spontaneously hypertensive rats
Acta Pharmacol Sin 25:763-768
236. Goebel M, Steckelings U M, **Unger Th** (2004)
 Was leisten ACE-Hemmer und AT1-Antagonisten jenseits der Blutdrucksenkung?
J Hypertonie 8:7-11
237. Hoheisel U, Reinöhl J, **Unger Th**, Mense S (2004)
 Acidic pH and capsaicin activate mechanosensitive group IV muscle receptors in the rat
Pain 110:149-157 (4.556)
238. Tian XL, Pinto YM, Costerousse O, Franz WM, Lippoldt A, Hoffmann S, **Unger Th**, Paul M (2004)
 Over-expression of angiotensin converting enzyme-1 augments cardiac hypertrophy in transgenic rats
Hum Mol Genet 13:1441-1450 (8.726)
239. Thöne-Reineke C, Zimmermann M, Neumann C, Krikov M, Li J, Gerova N, **Unger Th** (2004)
 Are angiotensin receptor blockers neuroprotective?
Curr Hypertens Rep 4:257-266
240. **Unger Th** (2004)
 Institut für Pharmakologie, Medizinische Fakultät der Christian-Albrechts-Universität zu Kiel (1994-2001)
 In: **Geschichte und Wirken der pharmakologischen, klinisch-pharmakologischen und toxikologischen Institute im deutschsprachigen Raum** (A. Philippu)
 Innsbruck: Berenkamp Buch- und Kunstverlag, S. 405-410
241. **Unger Th** (2004)
 Zur Kooperation Hochschule-Industrie
Med Klin 99, Suppl 1: 1-2
242. **Unger Th (Hrsg.)** (2004)
 Antithrombozytäre Strategien in der kardiovaskulären Akut- und Langzeittherapie
 Wissenschaftliches Symposium anlässlich der Verleihung des Robert-Koch-Award an Sanofi-Synthelabo, Berlin, 23. Januar 2004

243. Unger Th (Hrsg.) (2004)
Laudatio
Med Klin 99, Suppl 1: 26-27
 244. Bramlage P, Wittchen H-U, Pittrow D, Kirch W, Krause P, Lehnert H, Unger Th, Höfler M, Köpper B, Dahm S, Böhrer S, Sharma AM (2004)
Recognition and management of overweight and obesity in primary care in germany
Int. J Obesity 28:1299-1308, online doi:10.1038/sj.ijo.0802752 (2.794)
 245. Kaschina E., Stoll M, Sommerfeld M, Steckelings UM, Kreutz R, Unger Th (2004)
Genetic kininogen deficiency contributes to aortic aneurysm formation but not to atherosclerosis
Physiol Genomics 19:41-49 (4.667)
 246. Unger Th, Li J (2004)
The role of the rennin-angiotensin-aldosterone system in heart failure
J Renin Angiotensin Aldosterone Syst 5:Suppl 1: S7-10
 247. Bramlage P, Pittrow D, Wittchen H-U, Kirch W, Boehler S, Lehnert H, Hoeffler M, Unger Th, Sharma AM (2004)
Hypertension in overweight and obese primary care patients is highly prevalent and poorly controlled
Am J Hypertens 17:904-910 (3.103)
 248. Unger Th, McInnes GT, Neutel JM, Böhm M (2004)
The role of olmesartan medoxomil in the management of hypertension
Drugs 64:2731-2739 (4.611)
- 2005
249. Clasen R, Schupp M, Forst-Ludwig A, Sprang C, Clemenz M, Krikov M, Thöne-Reineke C, Unger Th, Kintscher U (2005)
PPAR γ -activating angiotensin Type-1 receptor blockers induce adiponectin
Hypertension 46:137-143 (5.342)
 250. Wilms H, Rosenstiel P, Unger Th, Deuschl G, Lucius R (2005)
Neuroprotection with angiotensin receptor antagonists: a review of the evidence and potential mechanisms
Am J Cardiovasc Drugs 5:245-253 (1.486)
 251. Kintscher U, Unger Th (2005)
Vascular protection in diabetes: a pharmacological view of angiotensin II type 1 receptor blockers
Acta Diabetol 42: 26-32 (0.335)
 252. Hoheisel U, Unger Th, Mense S (2005)
Excitatory and modulatory effects of inflammatory cytokines and neutrophins on mechanosensitive group IV muscle afferents in the rat
Pain 114:168-176 (4.061)
 253. Dragun D, Müller DN, Bräsen JH, Fritsche L, Nieminen-Kelhä M, Dechend R, Kintscher U, Rudolph B, Hoebcke J, Eckert D, Mazak I, Plehm R, Schönemann C, Unger Th, Budde K, Neumayer H-H, Luft FC, Wallukat G (2005)
Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection
New Engl J Med 352:558-569 (38.570)
 254. Li J, Culman J, Hörtnagl H, Zhao Y, Gerova N, Timm M, Blume A, Zimmermann M, Seidel K, Dirnagl U, Unger Th (2005)

Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury
FASEB J 19:617-619 (6.820)

255. **Unger Th, Parati G (2005)**
Acute stress and long-lasting blood pressure elevation: a possible cause of established hypertension?
J Hypertens 23:261-263 (4.871)
256. **Unger Th (2005)**
Does losartan reduce the risk of stroke in patients with hypertension?
Nature Clinical Practice Cardiovascular Medicine 2:236-237
257. **Kintscher U, Unger Th (2005)**
Does the AT2-receptor mediate anti-atherosclerotic actions?
J Hypertens 23:1469-1470 (4.871)
258. **Steckelings UM, Kaschina E, Unger Th (2005)**
The AT2 receptor-A matter of love and hate
Peptides 26:1401-1409 (2.511)
259. **Wruck CJ, Funke-Kaiser H, Pufe T, Kusserow H, Menk M, Scheff JH, Kruse ML, Stoll M, Unger Th (2005)**
Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated golgi protein
Arterioscler Thromb Vasc Biol 25:67-64 (7.432)
260. **Zhao, Y, Foryst-Ludwig A, Bruemmer D, Culman J, Bader M, Unger Th, Kintscher U (2005)**
Angiotensin II induces peroxisome proliferators-activated receptor gamma in PC12W cells via angiotensin type 2-receptor activation
J Neurochem 94:1395-1401 (4.824)
261. **Blume A, Undeutsch C, Zhao Y, Kaschina E, Culman J, Unger Th (2005)**
Ang II induces expression of inducible transcription factors of AP-1 and Krox families in rat brain
Am J Physiol Regul Integr Comp Physiol 289: R 845-50 (3.405)
262. **Schupp M, Unger Th (2005)**
Telmisartan: from lowering blood pressure to end-organ protection
Future Cardiology 1:7-15
263. **Doerfel N, Kaschina E, Unger Th (2005)**
Focus on various angiotensin II receptors in the treatment of hypertension
Renin Angiotensin Syst Cardiovasc Med 1(1):6-10
264. **Unger Th (2005)**
Editorial
Renin Angiotensin Syst Cardiovasc Med 1(1):1
265. **Unger Th (2005)**
Editorial
Renin Angiotensin Syst Cardiovasc Med 1(2):1
266. **Ruilope LM, Agabiti Rosei E, Bakris GL, Mancía G, Poulter NR, Taddei S, Unger Th, Volpe M, Waebler B, Zannad F (2005)**
Angiotensin receptor blockers: Therapeutic targets and cardiovascular protection
Blood Pressure 14:196-209 (1.027)
267. **Steckelings UM, Henz BM, Wiehstutz S, Unger Th, Artuc M (2005)**
Differential expression of angiotensin receptors in human cutaneous wound healing
Brit J Dermatol 153:887-893 (2.445)
268. **Xu J, Scholz A, Rösch N, Blume A, Unger Th, Kreutz R, Culman J, Gohlke P (2005)**

Low-dose lithium combined with captopril prevents stroke and improves survival in salt-loaded stroke-prone spontaneously hypertensive rats
J Hypertens 23:2277-2285 (4.871)

269. Sandmann S, [REDACTED] Fritzenkötter C, Spormann J, Tiede K, Fischer J, **Unger Th**
 Inhibition of the acute inflammatory response in the infarcted myocardium – comparison of AT1 receptor blockade versus ACE inhibition
 submitted
270. Yang L, **Unger Th**
 Modulation of delayed rectifier potassium current and L-type calcium current by glycyrrhetic acid in single Guinea-pig ventricular myocytes
 submitted 06/04
271. Schupp M, Clemenz M, Gineste R, Witt H, Janke J, Helleboid S, Hennuyer N, Ruiz P, **Unger Th**, Staels B, Kintscher U (2005)
 Molecular Characterization of New Selective PPAR γ Modulators with Angiotensin Receptor Blocking Activity
Diabetes 54:3442-3452 (8.848)
272. Schupp, M, Kintscher U, Fielitz J, Thomas J, Pregla R, Hetzer R, **Unger Th**, Regitz-Zagrosek V
 Cardiac PPAR α expression in patients with dilated cardiomyopathy
European Journal of Heart Failure (in press) (2.796)
273. Hoheisel U, **Unger Th**, Mense S (2005)
 The possible role of the NO-cGMP pathway in nociception: Different spinal and supraspinal action of enzyme blockers on rat dorsal horn neurones
Pain 117:358-367 (4.061)

[REDACTED]

- Hypertension, in press (U. Kintscher)
- Elena nach submitted fragen
- Jun Li nicht vergessen (s.o.)
- „In press“ steht immer über „submitted“
- Seitenumbruch !!
- Angabe über „alle Papers oder Reviewed Papers“
- Kein Dokument ohne Datum !!!
- Numerierung seit 1995 ist willkürlich. Sie macht nur Sinn, wenn man von Anfang an numeriert und dies Numerierung dann konsequent durchhält !

Please consider: Publikationslisten sind unsere Visitenkarten und das Einzige, was wir in der Forschung vorzuweisen haben !

Publication list of Prof. Thomas Unger, MD PhD

2002-2008. Reviewed papers

1. Blume A, Neumann C, Dorenkamp M, Culman J, Unger T. Involvement of adrenoceptors in the angiotensin II-induced expression of inducible transcription factors in the rat forebrain and hypothalamus. *Neuropharmacology*. 2002;42(2):281-288.
2. Culman J, Blume A, Gohlke P, Unger T. The renin-angiotensin system in the brain: possible therapeutic implications for AT(1)-receptor blockers. *J Hum Hypertens*. 2002;16(3):S64-70.
3. Dzau VJ, Bernstein K, Celermajer D, Cohen J, Dahlof B, Deanfield J, Diez J, Drexler H, Ferrari R, Van Gilst W, Hansson L, Hornig B, Husain A, Johnston C, Lazar H, Lonn E, Luscher T, Mancini J, Mimran A, Pepine C, Rabelink T, Remme W, Ruilope L, Ruzicka M, Schunkert H, Swedberg K, Unger T, Vaughan D, Weber M. Pathophysiologic and therapeutic importance of tissue ACE: a consensus report. *Cardiovasc Drugs Ther*. 2002;16(2):149-160.
4. Gohlke P, Kox T, Jurgensen T, von Kugelgen S, Rascher W, Unger T, Culman J. Peripherally applied candesartan inhibits central responses to angiotensin II in conscious rats. *Naunyn-Schmiedeberg's Arch Pharmacol*. 2002;365(6):477-483.
5. Gohlke P, Von Kugelgen S, Jurgensen T, Kox T, Rascher W, Culman J, Unger T. Effects of orally applied candesartan cilexetil on central responses to angiotensin II in conscious rats. *J Hypertens*. 2002;20(5):909-918.
6. Rosenstiel P, Gallinat S, Arlt A, Unger T, Sievers J, Lucius R. Angiotensin AT2 receptor ligands: do they have potential as future treatments for neurological disease? *CNS Drugs*. 2002;16(3):145-153.
7. Sandmann S, Prenzel F, Shaw L, Schauer R, Unger T. Activity profile of calpains I and II in chronically infarcted rat myocardium—influence of the calpain inhibitor CAL 9961. *Br J Pharmacol*. 2002;135(8):1951-1958.
8. Sandmann S, Spormann J, Prenzel F, Shaw L, Unger T. Calcium channel blockade limits transcriptional, translational and functional up-regulation of the cardiac calpain system after myocardial infarction. *Eur J Pharmacol*. 2002;453(1):99-109.
9. Sandmann S, Unger T. [Pathophysiological and clinical implications of AT(1)/AT(2) angiotensin II receptors in heart failure and coronary and renal failure]. *Drugs*. 2002;62(1): 43-52.
10. Scheifer C, Hoheisel U, Trudrung P, Unger T, Mense S. Rats with chronic spinal cord transection as a possible model for the at-level pain of paraplegic patients. *Neurosci Lett*. 2002;323(2):117-120.
11. Stoll M, Hahn AW, Jonas U, Zhao Y, Schieffer B, Fischer JW, Unger T. Identification of a zinc finger homeodomain enhancer protein after AT(2) receptor stimulation by differential mRNA display. *Arterioscler Thromb Vasc Biol*. 2002;22(2):231-237.
12. Unger T. The role of the renin-angiotensin system in the development of cardiovascular disease. *Am J Cardiol*. 2002;89(2A):3A-9A; discussion 10A.
13. Unger T. [New options in the drug treatment of hypertension: claim and reality]. *Dtsch Med Wochenschr*. 2002;127(45):2404-2406.
14. Friedrich MG, Dahlof B, Sechtem U, Unger T, Knecht M, Dietz R. Reduction (TELMAR) as assessed by magnetic resonance imaging in patients with mild-to-moderate hypertension—a prospective, randomised, double-blind comparison of telmisartan with metoprolol over a period of six months rationale and study design. *J Renin Angiotensin Aldosterone Syst*. 2003;4(4):234-243.

15. Groth W, Blume A, Gohlke P, Unger T, Culman J. Chronic pretreatment with candesartan improves recovery from focal cerebral ischaemia in rats. *J Hypertens.* 2003;21(11):2175-2182.
16. Hoheisel U, Scheifer C, Trudrung P, Unger T, Mense S. Pathophysiological activity in rat dorsal horn neurones in segments rostral to a chronic spinal cord injury. *Brain Res.* 2003;974(1-2):134-145.
17. Kaschina E, Unger T. Angiotensin AT1/AT2 receptors: regulation, signalling and function. *Blood Press.* 2003;12(2):70-88.
18. Kintscher U, Bruemmer D, Blaschke F, Unger T, Law RE. p38 MAP kinase negatively regulates angiotensin II-mediated effects on cell cycle molecules in human coronary smooth muscle cells. *Biochem Biophys Res Commun.* 2003;305(3):552-556.
19. Kintscher U, Unger T. Angiotensin II receptor expression: from maturation to pathogenesis. *Am J Physiol Regul Integr Comp Physiol.* 2003;285(1):R26-27.
20. Krause P, Wittchen HU, Kupper B, Pittrow D, Unger T, Sharma AM, Ritz E, Goke B, Lehnert H, Tschöpe C, Kirch W, Pfister H, Bramlage P, Hofler M. [Management of diabetes and hypertension: how do primary care physicians judge their performance?]. *Fortschr Med Orig.* 2003;121(1):12-18.
21. Lottermoser K, Unger T, Gohlke P, Vetter H, Dusing R. Differential effect of acute angiotensin II type 1 receptor blockade on the vascular and adrenal response to exogenous angiotensin II in humans. *Am J Hypertens.* 2003;16(6):445-452.
22. Pecs C, Unger T, Gohlke P. Effect of angiotensin AT2 receptor stimulation on vascular cyclic GMP production in normotensive Wistar Kyoto rats. *Int J Biochem Cell Biol.* 2003;35(6):963-972.
23. Reinecke K, Lucius R, Reinecke A, Rickert U, Herdegen T, Unger T. Angiotensin II accelerates functional recovery in the rat sciatic nerve in vivo: role of the AT2 receptor and the transcription factor NF-kappaB. *Faseb J.* 2003;17(14):2094-2096.
24. Reinohl J, Hoheisel U, Unger T, Mense S. Adenosine triphosphate as a stimulant for nociceptive and non-nociceptive muscle group IV receptors in the rat. *Neurosci Lett.* 2003;338(1):25-28.
25. Sandmann S, Kaschina E, Blume A, Kruse ML, Unger T. Bradykinin B1 and B2 receptors differentially regulate cardiac Na⁺-H⁺ exchanger, Na⁺-Ca²⁺ exchanger and Na⁺-HCO₃⁻ symporter. *Eur J Pharmacol.* 2003;458(1-2):3-16.
26. Scholze J, Unger T. [The ALLHAT study (antihypertensive and lipid-lowering treatment to prevent heart attack trial): primary antihypertensive agents - diuretics vs calcium channel blockers vs angiotensin-converting enzyme inhibitors]. *Internist (Berl).* 2003;44(9):1193-1195.
27. Tiede K, Stoter K, Petrik C, Chen WB, Ungefroren H, Kruse ML, Stoll M, Unger T, Fischer JW. Angiotensin II AT(1)-receptor induces biglycan in neonatal cardiac fibroblasts via autocrine release of TGFbeta in vitro. *Cardiovasc Res.* 2003;60(3):538-546.
28. Tschöpe C, Seidl U, Reinecke A, Riester U, Graf K, Schultheiss HP, Hilgenfeldt U, Unger T. Kinins are involved in the antiproteinuric effect of angiotensin-converting enzyme inhibition in experimental diabetic nephropathy. *Int Immunopharmacol.* 2003;3(3):335-344.
29. Unger T. The ongoing telmisartan alone and in combination with ramipril global endpoint trial program. *Am J Cardiol.* 2003;91(10A):28G-34G.
30. Unger T. Inhibiting angiotensin receptors in the brain: possible therapeutic implications. *Curr Med Res Opin.* 2003;19(5):449-451.
31. Unger T. [Diuretics—drug treatment of choice for uncomplicated hypertension?—contra]. *Dtsch Med Wochenschr.* 2003;128(47):2491-2492.

32. Unger T. [Awards for research and development of acarbose]. *Med Klin (Munich)*. 2003;98 Suppl 1:18-19.
33. Unger T. Blood pressure lowering and renin-angiotensin system blockade. *J Hypertens Suppl*. 2003;21(6):S3-7.
34. Unger T, Kaschina E. Drug interactions with angiotensin receptor blockers: a comparison with other antihypertensives. *Drug Saf*. 2003;26(10):707-720.
35. Wittchen HU, Krause P, Hofler M, Pfister H, Kupper B, Pittrow D, Bramlage P, Unger T, Sharma AM, Ritz E, Goke B, Lehnert H, Tschope D, Kirch W. [Aim, design and methods of the "Hypertension and diabetes screening and awareness" -- (HYDRA) study]. *Fortschr Med Orig*. 2003;121 Suppl 1:2-11.
36. Wittchen HU, Krause P, Hofler M, Pfister H, Ritz E, Goke B, Lehnert H, Tschope D, Kirch W, Pittrow D, Sharma AM, Bramlage P, Kupper B, Unger T. [Hypertension, diabetes mellitus and comorbidity in primary care]. *Fortschr Med Orig*. 2003;121(1):19-27.
37. Zhao Y, Biermann T, Luther C, Unger T, Culman J, Gohlke P. Contribution of bradykinin and nitric oxide to AT2 receptor-mediated differentiation in PC12 W cells. *J Neurochem*. 2003;85(3):759-767.
38. Bramlage P, Pittrow D, Wittchen HU, Kirch W, Boehler S, Lehnert H, Hoefler M, Unger T, Sharma AM. Hypertension in overweight and obese primary care patients is highly prevalent and poorly controlled. *Am J Hypertens*. 2004;17(10):904-910.
39. Bramlage P, Wittchen HU, Pittrow D, Kirch W, Krause P, Lehnert H, Unger T, Hofler M, Kupper B, Dahm S, Bohler S, Sharma AM. Recognition and management of overweight and obesity in primary care in Germany. *Int J Obes Relat Metab Disord*. 2004;28(10):1299-1308.
40. Hoheisel U, Reinohl J, Unger T, Mense S. Acidic pH and capsaicin activate mechanosensitive group IV muscle receptors in the rat. *Pain*. Jul 2004;110(1-2):149-157.
41. Kaschina E, Stoll M, Sommerfeld M, Steckelings UM, Kreutz R, Unger T. Genetic kininogen deficiency contributes to aortic aneurysm formation but not to atherosclerosis. *Physiol Genomics*. 2004;19(1):41-49.
42. Kusserow H, Unger T. Vasoactive peptides, their receptors and drug development. *Basic Clin Pharmacol Toxicol*. 2004;94(1):5-12.
43. Marshall RP, Gohlke P, Chambers RC, Howell DC, Bottoms SE, Unger T, McAnulty RJ, Laurent GJ. Angiotensin II and the fibroproliferative response to acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. 2004;286(1):L156-164.
44. Pittrow D, Kirch W, Bramlage P, Lehnert H, Hofler M, Unger T, Sharma AM, Wittchen HU. Patterns of antihypertensive drug utilization in primary care. *Eur J Clin Pharmacol*. 2004;60(2):135-142.
45. Schupp M, Janke J, Clasen R, Unger T, Kintscher U. Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity. *Circulation*. 2004;109(17):2054-2057.
46. Sharma AM, Wittchen HU, Kirch W, Pittrow D, Ritz E, Goke B, Lehnert H, Tschope D, Krause P, Hofler M, Pfister H, Bramlage P, Unger T. High prevalence and poor control of hypertension in primary care: cross-sectional study. *J Hypertens*. 2004;22(3):479-486.
47. Steckelings UM, Stoppelhaar M, Sharma AM, Wittchen HU, Krause P, Kupper B, Kirch W, Pittrow D, Ritz E, Goke B, Lehnert H, Tschope D, Hofler M, Pfister H, Unger T. HYDRA: possible determinants of unsatisfactory hypertension control in German primary care patients. *Blood Press*. 2004;13(2):80-88.

48. Thone-Reineke C, Zimmermann M, Neumann C, Krikov M, Li J, Gerova N, Unger T. Are angiotensin receptor blockers neuroprotective? *Curr Hypertens Rep.* 2004;6(4):257-266.
49. Tian XL, Pinto YM, Costerousse O, Franz WM, Lippoldt A, Hoffmann S, Unger T, Paul M. Over-expression of angiotensin converting enzyme-1 augments cardiac hypertrophy in transgenic rats. *Hum Mol Genet.* 2004;13(14):1441-1450.
50. Unger T. Lessons from ALLHAT. Are low budget diuretics first line therapy in hypertension? *Z Kardiol.* 2004;93(5):349-356.
51. Unger T. [Cooperation between university-industry]. *Med Klin (Munich).* Aug 15 2004;99 Suppl 1:1-2.
52. Unger T, Li J. The role of the renin-angiotensin-aldosterone system in heart failure. *J Renin Angiotensin Aldosterone Syst.* 2004;5(1):S7-10.
53. Unger T, McInnes GT, Neutel JM, Böhm M. The role of olmesartan medoxomil in the management of hypertension. *Drugs.* 2004;64(24):2731-2739.
54. Xia QG, Reinecke A, Dorenkamp M, Storz C, Bitterling H, Penz S, Cleutjens J, Daemen MJ, Simon R, Unger T. Comparison of cardioprotective effects of mibefradil and ramipril in stroke-prone spontaneously hypertensive rats. *Acta Pharmacol Sin.* 2004;25(6):763-768.
55. Zimmermann M, Unger T. Challenges in improving prognosis and therapy: the Ongoing Telmisartan Alone and in Combination with Ramipril Global End point Trial programme. *Expert Opin Pharmacother.* 2004;5(5):1201-1208.
56. Blume A, Undeutsch C, Zhao Y, Kaschina E, Culman J, Unger T. ANG III induces expression of inducible transcription factors of AP-1 and Krox families in rat brain. *Am J Physiol Regul Integr Comp Physiol.* 2005;289(3):R845-850.
57. Clasen R, Schupp M, Forst-Ludwig A, Sprang C, Clemenz M, Krikov M, Thone-Reineke C, Unger T, Kintscher U. PPARgamma-activating angiotensin type-1 receptor blockers induce adiponectin. *Hypertension.* 2005;46(1):137-143.
58. Dragun D, Müller DN, Brasen JH, Fritsche L, Nieminen-Kelha M, Dechend R, Kintscher U, Rudolph B, Hoebeke J, Eckert D, Mazak I, Plehm R, Schönmeyer C, Unger T, Budde K, Neumayer HH, Luft FC, Wallukat G. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. *N Engl J Med.* 2005;352(6):558-569.
59. Hoheisel U, Unger T, Mense S. Excitatory and modulatory effects of inflammatory cytokines and neurotrophins on mechanosensitive group IV muscle afferents in the rat. *Pain.* 2005;114(1-2):168-176.
60. Hoheisel U, Unger T, Mense S. The possible role of the NO-cGMP pathway in nociception: different spinal and supraspinal action of enzyme blockers on rat dorsal horn neurones. *Pain.* 2005;117(3):358-367.
61. Kintscher U, Unger T. Vascular protection in diabetes: a pharmacological view of angiotensin II type 1 receptor blockers. *Acta Diabetol.* 2005;42(1):S26-32.
62. Kintscher U, Unger T. Does the AT2-receptor mediate anti-atherosclerotic actions? *J Hypertens.* 2005;23(3):1469-1470.
63. Li J, Culman J, Hortnagl H, Zhao Y, Gerova N, Timm M, Blume A, Zimmermann M, Seidel K, Dimagli U, Unger T. Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury. *FASEB J.* 2005;19(6):617-619.

64. Ruilope LM, Rosci EA, Bakris GL, Mancía G, Poulter NR, Taddei S, Unger T, Volpe M, Waeber B, Zannad F. Angiotensin receptor blockers: therapeutic targets and cardiovascular protection. *Blood Press*. 2005;14(4):196-209.
65. Schupp M, Clemenz M, Gineste R, Witt H, Janke J, Helleboid S, Hennuyer N, Ruiz P, Unger T, Staels B, Kintscher U. Molecular Characterization of New Selective Peroxisome Proliferator-Activated Receptor (γ) Modulators With Angiotensin Receptor Blocking Activity. *Diabetes*. 2005;54(12):3442-3452.
66. Steckelings UM, Henz BM, Wiehstutz S, Unger T, Artuc M. Differential expression of angiotensin receptors in human cutaneous wound healing. *Br J Dermatol*. 2005;153(5):887-893.
67. Steckelings UM, Kaschina E, Unger T. The AT2 receptor—a matter of love and hate. *Peptides*. 2005;26(8):1401-1409.
68. Unger T. Does losartan reduce the risk of stroke in patients with hypertension? *Nat Clin Pract Cardiovasc Med*. 2005;2(5):236-237.
69. Unger T. [Laudatio. On the presentation of the 2005 Robert Koch Award to the drug tiotropium bromide (spiriva)]. *Med Klin (Munich)*. 2005;100 Suppl 1:17-18.
70. Unger T, Parati G. Acute stress and long-lasting blood pressure elevation: a possible cause of established hypertension? *J Hypertens*. 2005;23(2):261-263.
71. Wilms H, Rosenstiel P, Unger T, Deuschl G, Lucius R. Neuroprotection with angiotensin receptor antagonists: a review of the evidence and potential mechanisms. *Am J Cardiovasc Drugs*. 2005;5(4):245-253.
72. Wruck CJ, Funke-Kaiser H, Pufe T, Kusserow H, Menk M, Schefe JH, Kruse ML, Stoll M, Unger T. Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated Golgi protein. *Arterioscler Thromb Vasc Biol*. 2005;25(1):57-64.
73. Xu J, Scholz A, Rosch N, Blume A, Unger T, Kreutz R, Culman J, Gohlke P. Low-dose lithium combined with captopril prevents stroke and improves survival in salt-loaded, stroke-prone spontaneously hypertensive rats. *J Hypertens*. 2005;23(12):2277-2285.
74. Zhao Y, Foryst-Ludwig A, Bruemmer D, Culman J, Bader M, Unger T, Kintscher U. Angiotensin II induces peroxisome proliferator-activated receptor gamma in PC12W cells via angiotensin type 2 receptor activation. *J Neurochem*. 2005;94(5):1395-1401.
75. Clemenz M, Kintscher U, Unger T. The metabolic syndrome: cluster with a self-fulfilling loop? *J Hypertens*. 2006;24(2):257-258.
76. Goebel M, Clemenz M, Unger T. Effective treatment of hypertension by AT(1) receptor antagonism: the past and future of telmisartan. *Expert Rev Cardiovasc Ther*. 2006;4(5):615-629.
77. Hartge MM, Kintscher U, Unger T. Endothelial dysfunction and its role in diabetic vascular disease. *Endocrinol Metab Clin North Am*. 2006;35(3):551-560, viii-ix.
78. Sandmann S, Li J, Fritzenkotter C, Spormann J, Tiede K, Fischer JW, Unger T. Differential effects of olmesartan and ramipril on inflammatory response after myocardial infarction in rats. *Blood Press*. 2006;15(2):116-128.
79. Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's C (T) difference" formula. *J Mol Med*. 2006;84(11):901-10.
80. Schefe JH, Menk M, Reinemund J, Effertz K, Hobbs RM, Pandolfi PP, Ruiz P, Unger T, Funke-Kaiser H. A Novel Signal Transduction Cascade Involving Direct Physical Interaction of the

Renin/Prorenin Receptor With the Transcription Factor Promyelocytic Zinc Finger Protein. *Circ Res*. 2006;99(12):1355-66.

81. Schupp M, Kintscher U, Fielitz J, Thomas J, Pregla R, Hetzer R, Unger T, Regitz-Zagrosek V. Cardiac PPARalpha expression in patients with dilated cardiomyopathy. *Eur J Heart Fail*. 2006;8(3):290-294.
82. Schupp M, Lee LD, Frost N, Umbreen S, Schmidt B, Unger T, Kintscher U. Regulation of peroxisome proliferator-activated receptor gamma activity by losartan metabolites. *Hypertension*. 2006;47(3):586-589.
83. Thone-Reinecke C, Kalk P, Dorn M, Klaus S, Simon K, Pfab T, Godes M, Persson P, Unger T, Hoher B. High-protein nutrition during pregnancy and lactation programs blood pressure, food efficiency, and body weight of the offspring in a sex-dependent manner. *Am J Physiol Regul Integr Comp Physiol*. 2006;291(4):R1025-1030.
84. Unger T. [Innovation or pseudo-innovation: that is the question]. *Med Klin (Munich)*. 2006;101(3):257-262.
85. Xia QG, Reinecke A, Dorenkamp M, Daemen MJ, Simon R, Unger T. Effects of endothelin ET(A) receptor blocker LU 135250 on cardiac remodeling and survival in a hypertensive rat model of chronic heart failure. *Acta Pharmacol Sin*. 2006;27(11):1417-1422.
86. Hartge MM, Unger T, Kintscher U. The endothelium and vascular inflammation in diabetes. *Diab Vasc Dis Res*. 2007;4(2):84-88.
87. Hoheisel U, Unger T, Mense S. Sensitization of rat dorsal horn neurons by NGF-induced subthreshold potentials and low-frequency activation. A study employing intracellular recordings in vivo. *Brain Res*. 2007;1169:34-43.
88. Kintscher U, Bramlage P, Paar WD, Thoenes M, Unger T. Irbesartan for the treatment of hypertension in patients with the metabolic syndrome: A sub analysis of the Treat to Target post authorization survey. Prospective observational, two armed study in 14,200 patients. *Cardiovasc Diabetol*. 2007;6(1):12.
89. Li J, Doerffel Y, Hoher B, Unger T. Inflammation in the genesis of hypertension and its complications the role of angiotensin II. *Nephrol Dial Transplant*. 2007;22(11):3107-09.
90. Peters H, Unger T. Mast cells and the power of local RAS activation. *Nephrol Dial Transplant*. 2007;22(1):40-42.
91. Scholze J, Grimm B, Herrmann D, Unger T, Kintscher U. Optimal treatment of obesity-related hypertension: the Hypertension-Obesity-Sibutramine (HOS) study. *Circulation*. 2007;115(15):1991-1998.
92. Steckelings UM, Rettig R, Unger T. Angiotensin in the kidney: a key to understanding hypertension? *Cell Metab*. 2007;5(1):7-8.
93. Westermann D, Rutschow S, Jager S, Linderer A, Anker S, Riad A, Unger T, Schultheiss HP, Pauschinger M, Tschope C. Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of Angiotensin type I receptor antagonism. *Diabetes*. 2007;56(3):641-646.
94. Clemenz M, Frost N, Schupp M, Caron S, Foryst-Ludwig A, Bohm C, Hartge M, Gust R, Staels B, Unger T, Kintscher U. Liver-specific peroxisome proliferator-activated receptor alpha target gene regulation by the angiotensin type 1 receptor blocker telmisartan. *Diabetes*. 2008;57(5):1405-1413.

95. Foryst-Ludwig A, Clemenz M, Hohmann S, Hartge M, Sprang C, Frost N, Krikov M, Bhanot S, Barros R, Morani A, Gustafsson JA, Unger T, Kintscher U. Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma. *PLoS Genet.* 2008;4(6):1-16.
96. Kappert K, Kusserow H, Unger T. The pharmacological rationale behind polypharmacy in heart failure. *Heart Fail Monit.* 2008;6(1):20-27.
97. Kappert K, Unger T, Kintscher U. [Aliskiren hemifumarat]. *Dtsch Med Wochenschr.* 2008;133(24):1308-1312.
98. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky P, Barth TF, Dragun D, Skurk T, Hauner H, Blüher M, Unger T, Wolf AM, Knippschild U, Hombach V, Marx N. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler Thromb Vasc Biol.* 2008;28(7):1304-1310.
99. Krikov M, Thone-Reineke C, Müller S, Villringer A, Unger T. Candesartan but not ramipril pretreatment improves outcome after stroke and stimulates neurotrophin BDNF/TrkB system in rats. *J Hypertens.* 2008;26(3):544-552.
100. Scheffé JH, Unger T, Funke-Kaiser H. PLZF and the (pro)renin receptor. *J Mol Med.* 2008;86(6):623-627.
101. Schmerbach K, Scheffé JH, Krikov M, Müller S, Villringer A, Kintscher U, Unger T, Thone-Reineke C. Comparison between single and combined treatment with candesartan and pioglitazone following transient focal ischemia in rat brain. *Brain Res.* 2008;1208:225-233.
102. Werner C, Baumhakel M, Teo KK, Schmieder R, Mann J, Unger T, Yusuf S, Böhm M. RAS blockade with ARB and ACE inhibitors: current perspective on rationale and patient selection. *Clin Res Cardiol.* 2008. in press.
103. Yusuf S, Teo KK, Pogue J, Dyal L, Copland I, Schumacher H, Dagenais G, Sleight P, Anderson C. Telmisartan, ramipril, or both in patients at high risk for vascular events. *N Engl J Med.* 2008;358(15):1547-1559.
104. Scheffé J, Neumann C, Goebel M, Danser J, Kirsch S, Gust R, Kintscher U, Unger T, Funke-Kaiser H. Prorenin engages the (pro)renin receptor like renin and both ligand activities are unopposed by aliskiren. *J Hypertens.* 2008; 26(9):1787-1794.
105. Bakris G, Böhm M, Dagenais G, Diener HC, Fujita T, Gorelick P, Kjeldsen SE, Laakso M, Mancina G, Pitt B, Sharma A, Sleight P, Teo K, Unger T, Weber M, Williams B, Zannad F. Cardiovascular protection for all individuals at high risk: evidence-based best practice. *Clin Res Cardiol.* 2008 Aug 23. [Epub ahead of print].
106. The Telmisartan Randomised Assessment Study in ACE intolerant subjects with cardiovascular Disease (TRANSCEND) Investigators. Effects of the angiotensin-receptor blocker telmisartan on cardiovascular events in high-risk patients intolerant to angiotensin-converting enzyme inhibitors: a randomised controlled trial. 2008. *The Lancet*, In Press, Corrected Proof, Available online 29 August 2008.
107. Kintscher U, Foryst-Ludwig A, Unger T. Inhibiting angiotensin type 1 receptors as a target for diabetes. *Expert Opin Ther Targets.* Oct 2008;12(10):1257-1263.
108. Kaschina E, Schrader F, Sommerfeld M, Kemnitz UR, Grzesiak A, Krikov M, Unger T. Telmisartan prevents aneurysm progression in the rat by inhibiting proteolysis, apoptosis and inflammation. *J Hypertens.* 2008 Dec;26(12):2361-73.

109. Thöne-Reineke C, Neumann C, Namsolleck P, Schmerbach K, Krikov M, Schemm JH, Lucht K, Hörtnagl H, Godes M, Müller S, Rumschüssel K, Funke-Kaiser H, Villringer A, Steckelings UM, Unger T. The beta-lactam antibiotic, ceftriaxone, dramatically improves survival, increases glutamate uptake and induces neurotrophins in stroke. *J Hypertens*. 2008 Dec;26(12):2426-35.
110. Kaschina E, Grzesiak A, Li J, Foryst-Ludwig A, Timm M, Rompe F, Sommerfeld M, Kemnitz UR, Curato C, Namsolleck P, Tschöpe C, Hallberg A, Alterman M, Hucko T, Paetsch I, Dietrich T, Schnackenburg B, Graf K, Dahlöf B, Kintscher U, Unger T, Steckelings UM. Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction? *Circulation*. 2008 Dec 9;118(24):2523-32.

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT B

Angiotensin II Type 2 Receptor Stimulation

A Novel Option of Therapeutic Interference With the Renin-Angiotensin System in Myocardial Infarction?

Elena Kaschina, MD*; Aleksandra Grzesiak, MD*; Jun Li, MD; Anna Foryst-Ludwig, PhD;

Melanie Timm, MTA; Franziska Rompe, MSc; Manuela Sommerfeld, MTA;

U. Rudolf Kemnitz, MTA; Caterina Curato, MSc; Pawel Namsolleck, MSc;

Carsten Tschöpe, MD, PhD; Anders Hallberg, PhD; Mathias Alterman, PhD; Thomas Hucko, MD; Ingo Paetsch, MD, PhD; Thore Dietrich, PhD; Bernhard Schnackenburg, PhD; Kristof Graf, MD, PhD; Björn Dahlöf, MD, PhD; Ulrich Kintscher, MD, PhD; Thomas Unger, MD, PhD*; U. Muscha Steckelings, MD*

Background—This study is the first to examine the effect of direct angiotensin II type 2 (AT_2) receptor stimulation on postinfarct cardiac function with the use of the novel nonpeptide AT_2 receptor agonist compound 21 (C21).

Methods and Results—Myocardial infarction (MI) was induced in Wistar rats by permanent ligation of the left coronary artery. Treatment with C21 (0.01, 0.03, 0.3 mg/kg per day IP) was started 24 hours after MI and was continued until euthanasia (7 days after MI). Infarct size was assessed by magnetic resonance imaging, and hemodynamic measurements were performed via transthoracic Doppler echocardiography and intracardiac Millar catheter. Cardiac tissues were analyzed for inflammation and apoptosis markers with immunoblotting and real-time reverse transcription polymerase chain reaction. C21 significantly improved systolic and diastolic ventricular function. Scar size was smallest in the C21-treated rats. In regard to underlying mechanisms, C21 diminished MI-induced Fas-ligand and caspase-3 expression in the peri-infarct zone, indicating an antiapoptotic effect. Phosphorylation of the p44/42 and p38 mitogen-activated protein kinases, both involved in the regulation of cell survival, was strongly reduced after MI but almost completely rescued by C21 treatment. Furthermore, C21 decreased MI-induced serum monocyte chemoattractant protein-1 and myeloperoxidase as well as cardiac interleukin-6, interleukin-1 β , and interleukin-2 expression, suggesting an antiinflammatory effect.

Conclusions—Direct AT_2 receptor stimulation may be a novel therapeutic approach to improve post-MI systolic and diastolic function by antiapoptotic and antiinflammatory mechanisms. (*Circulation*. 2008;118:2523-2532.)

Key Words: angiotensin ■ angiotensin AT_2 receptor ■ myocardial infarction ■ pharmacology

Controversy has always existed about the angiotensin II type 2 receptor (AT_2R). Initially, after the first description of the AT_2R , it was assumed that the AT_2R may be without any physiological function. It was not until 5 years after its identification that a first physiological function, antiproliferation, could be ascribed to the AT_2R .^{1,2} Today, it is widely accepted that, in many instances, the AT_2R counteracts angiotensin II type 1 receptor (AT_1R)-mediated actions, which in most cases is beneficial for the organism.³

Clinical Perspective p 2532

A number of animal studies and the Valsartan in Acute Myocardial Infarction (VALIANT) trial have shown that AT_1R blockers improve survival and cardiac function after myocardial infarction (MI).⁴ It has been speculated that the AT_2R contributes to this effect because AT_1R blockade leads to an increased synthesis of angiotensin II, which in turn stimulates the unopposed AT_2Rs . However, results of animal

Received April 9, 2008; accepted September 12, 2008.

From the Center for Cardiovascular Research, Institute of Pharmacology, Charité-Universitätsmedizin Berlin, Berlin, Germany (E.K., A.G., J.L., A.F.-L., M.T., F.R., M.S., U.R.K., C.C., P.N., U.K., T.U., U.M.S.); Department of Cardiology and Pneumology, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany (C.T.); Department of Medicinal Chemistry, Organic Pharmaceutical Chemistry, Uppsala University, Uppsala, Sweden (A.H., M.A.); Deutsches Herzzentrum Berlin, Klinik für Innere Medizin—Kardiologie, Berlin, Germany (T.H., I.F., T.D., K.G.); Philips Clinical Science, Hamburg, Germany (B.S.); and Sahlgrenska University Hospital/Östra, Göteborg, Sweden (B.D.).

*Dr. Kaschina, Grzesiak, Unger, and Steckelings contributed equally to this work.

The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.784868/DC1>. Correspondence to: Dr. Thomas Unger, Center for Cardiovascular Research, Institute of Pharmacology, Charité-Universitätsmedizin Berlin, Hassoche Strasse 3-4, 10115 Berlin, Germany. E-mail: thomas.unger@charite.de

© 2008 American Heart Association, Inc.

Circulation is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.108.784868

studies directly addressing the role of the AT₂R in MI are again controversial. Although most studies claim that the AT₂R improves postinfarct cardiac function, others report either no effect on the outcome or even deterioration.⁵

The inconsistency of data about AT₂R actions may be due in part to the fact that detection and assignment of AT₂R-mediated effects have always been difficult. AT₂R-mediated effects had to be examined either by treatment of cells or animals with angiotensin II under concomitant AT₁R blockade, which resulted in complex experimental protocols, or they were examined in genetically altered animals either overexpressing or lacking the AT₂R. The only currently available pharmacological AT₂R antagonist, PD12319, lacks selectivity when applied at higher doses and has thus given rise to contradictory findings, especially when applied *in vivo*.⁶ An experimental tool to directly stimulate the AT₂R under *in vivo* conditions was lacking until now.

In 2004, the synthesis of the first nonpeptide AT₂R agonist (compound 21 [C21]) was published, a compound that allows investigators to specifically and selectively stimulate the AT₂R *in vitro* and *in vivo* settings.⁷ With a K_i of 0.4 nmol/L for the AT₂R and a K_i > 10 μ mol/L for the AT₁R, this substance possesses high selectivity for the AT₂R.

The present study is the first to examine the role of the AT₂R in post-MI cardiac function by direct AT₂R stimulation *in vivo* with the use of the nonpeptide AT₂R agonist C21. MI was induced in normotensive Wistar rats by permanent ligation of the anterior descending artery. After a 1-week treatment period, scar volume was assessed by magnetic resonance imaging, and hemodynamic function was assessed by echocardiography and Millar catheter. In a first attempt to address the underlying mechanisms of C21 actions, markers of inflammation and apoptosis were determined in cardiac tissue and plasma. To obtain a first qualitative estimate of the effectiveness of C21, we included a group of animals treated with the AT₁R antagonist candesartan as a reference drug.

Methods

Animals

Male normotensive Wistar rats (weight, 200 to 220 g; Harlan Winkelmann, Borcheln, Germany) were kept in a specific pathogen-free barrier under standardized conditions with respect to temperature and humidity and were housed on a 12-hour light/12-hour dark cycle in groups of 5 with food and water *ad libitum*. Animal housing, care, and applications of experimental procedures complied with the *Guide for the Care and Use of Laboratory Animals* of the State Government of Berlin, Germany.

Myocardial Infarction

Rats were anesthetized with ketamine/xylazine (Sigma Aldrich Chemie, Steinheim, Germany) 80 mg/10 mg/kg IP, intubated, and ventilated with a small-animal ventilator (Starling Ideal Ventilator, Harvard Apparatus) with room air at a rate of 75 cycles per minute and a tidal volume of 300 μ L. A left lateral thoracotomy was performed, and a suture was tightened around the proximal left anterior descending coronary artery. Sham-operated rats underwent the same surgical procedure with the exception of coronary ligation.

Experimental Protocol

Trans thoracic Doppler echocardiography was performed 24 hours after MI to assess post-MI baseline cardiac function. For better comparability of outcome between groups, rats were assigned to 2

different groups for later data analysis according to ejection fraction (EF): (1) rats with EF >35% and (2) rats with EF \leq 35%. All data presented in this study apply to animals with EF >35% unless indicated otherwise. After being allocated to a certain category with respect to EF, animals were further randomly assigned to the following treatments: (1) vehicle; (2) C21 (0.01; 0.03; 0.3 mg/kg per day); C21 was kindly provided by A. Hallberg, University of Uppsala, Uppsala, Sweden; C21 is now available from Vicore Pharma, Göteborg, Sweden; (3) C21+PD12319 (0.03 mg/kg C21; 3 mg/kg PD12319); (4) candesartan (0.1 mg/kg per day); or (5) C21+candesartan (0.03 mg/kg C21; 0.1 mg/kg per day candesartan). A low dose of candesartan (0.1 mg/kg per day) was chosen because in prior experiments of our group, this dose had shown beneficial functional and cellular effects after stroke without affecting blood pressure. Drugs were applied intraperitoneally once daily starting 24 hours after MI to allow for prior determination of EF under untreated conditions. After 7 days of treatment, animals were euthanized, and plasma and hearts were collected. Sham-operated animals served as controls. The number of animals per group was 12 at the time of euthanasia. To achieve this group size, a total of 298 rats underwent successful coronary occlusion.

Trans thoracic Doppler Echocardiography

Trans thoracic Doppler echocardiography (M-mode and Doppler measurements) was performed on days 1 and 7 after MI in anesthetized rats with the use of the high-resolution imaging system Vevo 770 (VisualSonics Inc, Toronto, Canada).⁸ For details, see the online-only Data Supplement.

Determination of Left Ventricular and Scar Volumes by Magnetic Resonance Imaging

Cardiac-triggered cine and scar magnetic resonance imaging was performed on a conventional clinical 3.0-T scanner (Philips Achieva CV 3.0 T, Best, Netherlands) equipped with a QuasarDual gradient system (80 mT/m; 200 mT/m per millisecond slow rate) and specifically designed software (release 2.5.1 with implementation of a small-animal software patch developed by GyroTools Ltd, Zurich, Switzerland). For details, see the online-only Data Supplement.

Analysis of Hemodynamic Parameters

On day 7 after MI, before euthanasia, rats were anesthetized with ketamine/xylazine (80 mg/10 mg/kg IP; Sigma). An SPR-407 Mikro-Tip pressure catheter transducer (Millar Instruments, Houston, Tex) was passed through the right carotid artery and inserted into the aorta for the recording of heart rate and arterial pressure under constant pressure monitoring. The catheter was then advanced into the left ventricle for measurement of cardiac parameters. The catheter was connected via PowerLab/4 U (ADInstruments, Spechbach, Germany) to a computer running MacLab (Chart 5 software). Data were recorded over 10 minutes, stored, and later analyzed with Chart software (Blood Pressure Module).

Enzyme-Linked Immunosorbent Assay, Quantitative Real-time Reverse Transcription Polymerase Chain Reaction, and Western Blot Analysis

Enzyme-linked immunosorbent assays for the determination of plasma monocyte-chemoattractant protein-1 and myeloperoxidase, real-time reverse transcription polymerase chain reactions for the measurement of interleukin (IL)-1 β , IL-2, and IL-6 in cardiac tissue as well as H9C2 cells, and immunoblotting for the quantification of cardiac caspase-3, Fas ligand, phospho-p38 and phospho-p44/42 mitogen-activated protein kinases (MAPKs) were performed according to standard protocols. Details, primers, and antibodies can be found in the Methods section of the online-only Data Supplement.

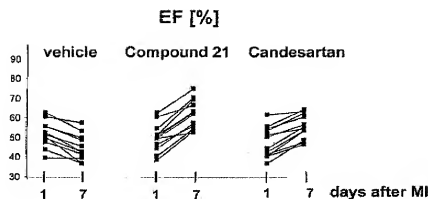


Figure 1. Change of EF in individual animals from treatment start (day 1) to end of treatment (day 7).

Cell Culture Experiments

Neonatal rat cardiomyocytes (H9C2 cell line) were incubated for 10 hours in high-glucose Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and treated with 10 ng/mL recombinant tumor necrosis factor- α (TNF- α) to induce cytokine expression and mimic the post-MI inflammatory response in the heart. Incubation was performed with or without coinubation with 10^{-6} mol/L C21 or C21 plus 10^{-3} mol/L PD123319.

Statistical Analysis

Results were expressed as mean \pm SEM. Multiple comparisons were analyzed with 1-way ANOVA followed by the Bonferroni post hoc test. Two-group comparisons were analyzed by the 2-tailed Student unpaired *t* test for independent samples. Differences were considered statistically significant at the value of $P < 0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Basal Parameters

Postinfarct mortality was 31%. Animal losses all occurred within the first 24 hours after MI before first echocardiography and before animals were assigned to certain treatment groups. No animals died on days 2 to 7 after MI. Mean EF was equal in all treatment groups at treatment start (Figure 1). Body and heart weight did not differ between treatment groups or between animals with or without MI (data not shown).

Hemodynamic Measurements

Hemodynamic parameters obtained on day 7 after MI/sham by Millar catheter in animals with EF $> 35\%$ after infarct are presented in Table 1 and Figure 2; those obtained in animals with severe impairment of cardiac function (EF $\leq 35\%$) are presented in Table I of the online-only Data Supplement.

Heart rate, ventricular systolic pressure, and systolic and diastolic blood pressure were not significantly altered by any treatment regimen or surgical procedure. Infarcted animals presented with a significant decrease in contractility as well as in maximal (dP/dt_{max}) and minimal (dP/dt_{min}) peak rate of left ventricular pressure. Moreover, left ventricular end-diastolic pressure (LVEDP) was significantly increased as a result of MI. In vivo treatment of rats with the AT₂R agonist C12 (0.03 or 0.3 mg/kg body wt) or with the AT₂R antagonist candesartan (0.1 mg/kg body wt) significantly enhanced contractility, dP/dt_{max} , and dP/dt_{min} , whereas LVEDP was reduced. The AT₂R antagonist PD123319 attenuated the effect of C21 on contractility and in part, but not significantly, on dP/dt_{min} and LVEDP but not on dP/dt_{max} . The combination of C21 with candesartan was not superior to C21 monotherapy.

In animals with severe postinfarct impairment of cardiac function (EF $\leq 35\%$), C21 (0.03 mg/kg body wt) still led to a significant improvement of LVEDP, contractility, dP/dt_{max} , and dP/dt_{min} (Table I of the online-only Data Supplement).

Table 1. Hemodynamic Parameters Measured by Millar Catheter 7 Days After MI

	Sham (n=12)	Vehicle (n=12)	C21 0.03 mg/kg (n=12)	C21 0.3 mg/kg (n=12)	Candesartan 0.1 mg/kg (n=12)	C21 + PD123319 (n=12)	C21 + Candesartan (n=12)
VSP, mm Hg	128 \pm 5	115 \pm 3	124 \pm 4	112 \pm 5	115 \pm 6	117 \pm 7	112 \pm 5
LVEDP, mm Hg	7.5 \pm 0.6	22.9 \pm 1.5*	13 \pm 1.6	9.0 \pm 1.3	18.8 \pm 1.7†	15.7 \pm 1.0‡	12.4 \pm 1.7
Contractility index, 1/s	106 \pm 2.7	67 \pm 2.9*	98 \pm 2.5§	97 \pm 3.8§	81 \pm 2.3	83 \pm 1.5#	95.2 \pm 3.4§
dP/dt_{max} , mm Hg/s	7532 \pm 321	3864 \pm 114*	6667 \pm 235§	6850 \pm 261§	5193 \pm 278†	4864 \pm 214‡	6280 \pm 319§
dP/dt_{min} , mm Hg/s	6795 \pm 231	2967 \pm 191*	5129 \pm 277‡§	5196 \pm 309§	3385 \pm 163*	3861 \pm 214†	4805 \pm 315
SBP, mm Hg	122 \pm 6	115 \pm 3	124 \pm 6	115 \pm 5	115 \pm 7	117 \pm 4	114 \pm 6
DBP, mm Hg	88 \pm 2	83 \pm 3	88 \pm 3	81 \pm 4	80 \pm 6	84 \pm 3	84 \pm 5
Pulse pressure, mm Hg	33 \pm 2	32 \pm 2	37 \pm 3	34 \pm 3	35 \pm 2	33 \pm 3	30 \pm 3
Heart rate, bpm	238 \pm 5	258 \pm 19	232 \pm 10	248 \pm 6	252 \pm 10	240 \pm 9	246 \pm 10

VSP indicates ventricular systolic pressure; SBP, systolic blood pressure; and DBP, diastolic blood pressure.

* $P < 0.005$ vs sham; † $P < 0.005$ vs sham; ‡ $P < 0.05$ vs sham; § $P < 0.0005$ vs vehicle; || $P < 0.005$ vs vehicle; ‡ $P < 0.05$ vs vehicle; # $P < 0.05$ vs C21.

Millar catheter

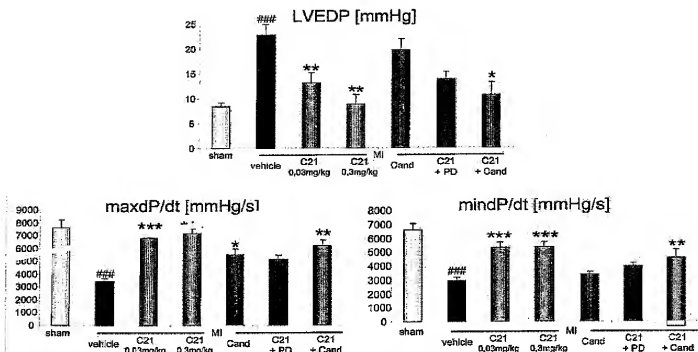


Figure 2. Hemodynamic parameters measured by Millar catheter. PD indicates PD123319; Cand, candesartan. ^{###} $P < 0.005$ vs sham; ^{*} $P < 0.0005$ vs vehicle; ^{**} $P < 0.005$ vs vehicle; ^{*} $P < 0.05$ vs vehicle.

Transthoracic Doppler Echocardiography

Measurements by transthoracic Doppler echocardiography confirmed that no changes in heart rate could be found between groups (Table 2; Table II in the online-only Data Supplement).

MI caused a significant increase in systolic (LVSDs) and diastolic left ventricular inner diameter (LVDD) and peak velocity of early filling wave (E), whereas fractional shortening, EF, peak velocity of late filling wave (A), and E-wave deceleration time (EDT) were significantly reduced (Table 2; Figure 3). Treatment with C21 (0.03 or 0.3 mg/kg body wt; only 0.03 mg/kg body wt in rats with EF

$\leq 35\%$) or with candesartan (0.1 mg/kg body wt) led to a significant improvement of all of these parameters with the only exceptions being LVDD and A in rats with EF $\leq 35\%$. The pronounced effect of treatment with C21 on left ventricular wall kinesis and peak velocity of late filling wave is also clearly recognizable on the representative echocardiographic images shown in Figure 4A and 4B.

The AT₁R antagonist PD123319 completely or partly abolished the effects of C21 on LVSDs and LVDD, fractional shortening, EF, E, A, E/A, and EDT (Table 2; Figure 3). Again, the combination of C21 and candesartan did not elicit

Table 2. Transthoracic Doppler Echocardiography Measurements 7 Days After MI

	Sham (n=12)	Vehicle (n=12)	C21 0.03 mg/kg (n=12)	C21 0.3 mg/kg (n=12)	Candesartan 0.1 mg/kg (n=12)	C21 + PD123319 (n=12)	C21 + Candesartan (n=12)
LVDD, mm	7.1±0.14	8.7±0.2†	7.4±0.11§	7.5±0.11§	7.6±0.09‡	8.1±0.3*	7.7±0.25¶
LVDS, mm	3.8±0.11	6.3±0.2‡	4.5±0.18‡	4.3±0.12§	5.2±0.2‡	6.0±0.3‡**	4.7±0.27‡§
Fractional shortening, %	46.3±1.2	26.1±1.6‡	39.0±1.8‡§	35.1±1.7‡	34.4±1.6‡	22.6±1.5‡**	34.2±3.1¶
EF, %	77±1.1	49±2.0‡	67±2.1‡§	63±2.1‡	61±2.1‡	43±2.4‡#	62.3±3.7‡
E, mm/s	1099±27	1580±29‡	1197±27§	1040±26§	1219±42§	1437±45‡#	1299±33‡§
A, mm/s	629±41	271±24‡	586±40§	517±31‡	571±45‡	285±44‡#	576±43‡
E/A	1.7±0.06	5.7±0.4‡	2±1*	2.2±0.08*	2.1±0.07*	5.0±1.2‡**	2.2±0.5‡
EDT, ms	26±0.8	15±0.5‡	22±1¶	20±0.5¶	21±1.5¶	16.5±0.7‡#	20±0.8‡
Heart rate, bpm	372±14	386±9	374±7	380±13	378±10	394±11	388±10

* $P < 0.05$ vs sham; † $P < 0.001$ vs sham; ‡ $P < 0.0001$ vs sham; § $P < 0.0001$ vs vehicle; ¶ $P < 0.001$ vs vehicle; # $P < 0.05$ vs vehicle; * $P < 0.0001$ vs C21; ** $P < 0.001$ vs C21.

Echocardiography

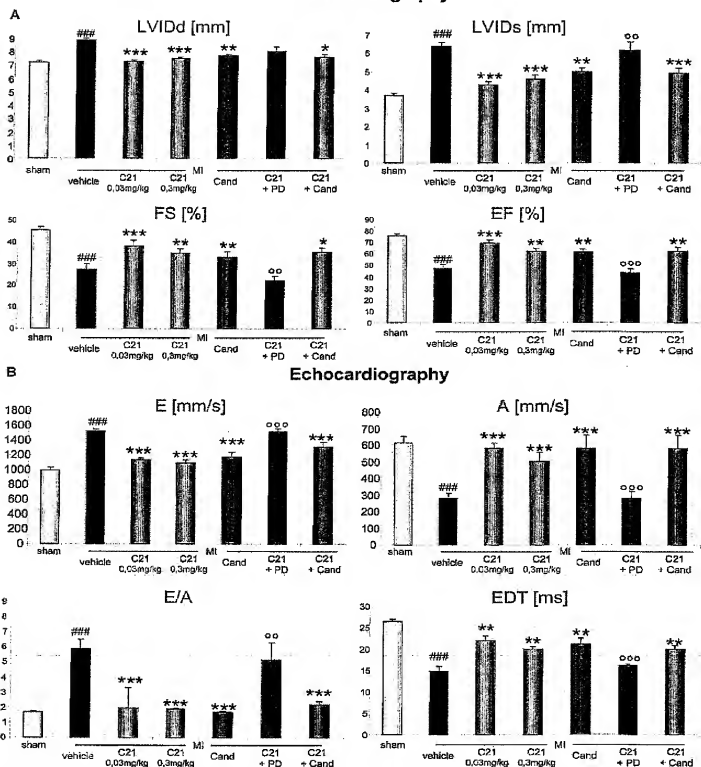


Figure 3. Hemodynamic parameters measured by echocardiography. PD indicates PD123319; Cand, candesartan. ### $P < 0.0001$ vs sham; *** $P < 0.0001$ vs vehicle; ** $P < 0.001$ vs vehicle; * $P < 0.05$ vs vehicle; °° $P < 0.0001$ vs C21; °°° $P < 0.001$ vs C21; °°°° $P < 0.0001$ vs C21.

any synergistic or additional effects. Whereas Figure 3 illustrates differences in outcome between treatment groups, the impact of different treatment regimens (vehicle, C21, candesartan) on EF in individual animals is shown in Figure

1 (and Figure I in the online-only Data Supplement for animals with EF $\leq 35\%$).

The effect of C21 on postinfarct cardiac function was dose dependent. Although 0.01 mg/kg body wt was widely ineffective

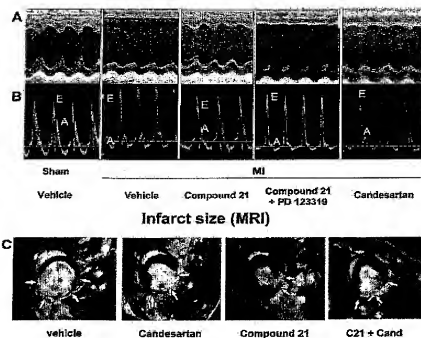


Figure 4. A, Representative M-mode echocardiograms. Note left ventricular dilatation, thinning, and akinesis of the anterior wall as well as hypokinesis of the posterior wall in vehicle-treated rats after MI. C21 and candesartan prevented left ventricular dilatation and improved anterior and posterior wall motion. The effect of C21 was abolished by PD123319 cotreatment. B, Representative pulsed-wave Doppler spectra of mitral inflow. Note increased E velocity, rapid deceleration of E wave, and decreased A wave velocity in vehicle-treated rats after MI. C21 and candesartan treatment decreased E velocity, slowed deceleration of E wave, and restored A wave velocity. The effect of C21 was abolished by PD123319 cotreatment. C, Representative cine magnetic resonance images showing delayed enhancement magnetic resonance imaging (MRI). Cand indicates candesartan. White arrows show segmental extent of scar tissue.

(data not shown), cardiac function significantly improved with 0.03 mg/kg body wt; further improvement was only slight, and did not occur in all cases, in animals treated with 0.3 mg/kg body wt (Tables 1 and 2).

Magnetic Resonance Imaging

Magnetic resonance imaging at day 7 after MI/sham revealed that in the C21- but not in the candesartan-treated animals, scar size was significantly smaller than in vehicle-treated rats (Table 3, Figure 4C). Furthermore, the combination of C21 plus candesartan, and, to a lesser extent, candesartan alone significantly improved EF (Table 3).

Inflammation Markers

Inflammation markers were measured (1) in plasma, (2) in cardiac tissue *ex vivo*, and (3) in fetal cardiomyocytes *in vitro*, as follows.

1. Plasma monocyte chemoattractant protein-1 (MCP-1) and myeloperoxidase were significantly increased in rats after MI (Figure 5). In rats treated with C21, a significant reduction in MCP-1 and myeloperoxidase levels could be noticed compared with vehicle-treated animals.
2. The proinflammatory cytokines IL-1 β , IL-2, and IL-6 were determined by real-time reverse transcription polymerase chain reaction in the peri-infarct zone. Expression of all 3 cytokines was increased in hearts of infarcted, vehicle-treated animals (Figure 6). C21 treatment significantly reduced expression levels of IL-1 β , IL-2, and IL-6 back to baseline levels or even below. In

this experimental setting, C21 proved to be as effective as the AT₂R antagonist candesartan. The effect of C21 could be inhibited by cotreatment of animals with the AT₂R antagonist PD123319.

3. To show that the antiinflammatory effects of C21 were not secondary to any hemodynamic changes or to reduced infarct size, the antiinflammatory properties of C21 were corroborated in the fetal cardiomyocyte cell line H9C2. Cells were incubated for 10 hours with TNF- α (10 ng/mL) to mimic the inflammatory response after MI. TNF- α treatment led to a significant increase in IL-6 expression in these cells (Figure 6D). Cotreatment of cells with C21 (10^{-6} mol/L) significantly reduced TNF- α -induced IL-6 expression. This antiinflammatory effect of C21 was inhibited by the AT₂R antagonist PD123319 (10^{-5} mol/L).

Apoptosis Marker

The apoptosis markers Fas ligand and caspase-3 were measured in cardiac tissue (peri-infarct zone) by immunoblotting (Figure 7). MI led to a significant increase in the expression of both markers. C21 and candesartan reduced this increased expression to a comparable extent. The effect of C21 was blocked by the AT₂R antagonist PD123319.

p44/42 MAPK, p38 MAPK

One week after MI, the cardiac expression of phosphorylated p44/42 MAPK and p38 MAPK was reduced to undetectable levels in vehicle-treated rats (Figure 8). Whereas candesartan

Table 3. Magnetic Resonance Tomography 7 Days After MI

	Sham (n=10)	Vehicle (n=10)	C21 0.03 mg/kg (n=10)	Candesartan 0.1 mg/kg (n=10)	C21+Candesartan (n=10)
EF, %	66.75 \pm 0.4	31.7 \pm 2.1*	54.4 \pm 1.9†	42.6 \pm 3†	51.3 \pm 3†
Scar volume, %		20.5 \pm 1.5	14.6 \pm 1.4†	20.7 \pm 1.4	18.4 \pm 2.3

* $P<0.0001$ vs sham; † $P<0.05$ vs vehicle; ‡ $P<0.0005$ vs vehicle.

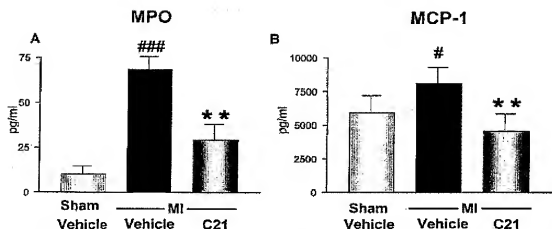


Figure 5. Myeloperoxidase (MPO) (A) and MCP-1 (B) levels in plasma estimated by enzyme-linked immunosorbent assay. MI led to a significant increase in myeloperoxidase and MCP-1 levels, which could be significantly reduced by C21 (0.03 mg/kg per day IP (MPO: ### P <0.001 vs vehicle, ** P <0.01 vs C21; MCP-1: # P <0.05 vs vehicle, ** P <0.01 vs C21; n =6 per group).

treatment led to a slight increase in the expression of both MAPKs, C21 restored phospho-p44/42 and phospho-p38 MAPK expression back to levels seen in sham-operated, noninfarcted animals. This effect of C21 was almost completely blocked by cotreatment with the AT₂R antagonist PD123319.

Discussion

Although it is unanimously accepted that stimulation of AT₂R contributes to tissue damage, inflammation, and un-

favorable tissue remodeling after MI, data about the role of the AT₂R in this context are controversial.³ This is certainly owed to the fact that, until now, practicable and reliable tools to study AT₂R actions by direct AT₂R stimulation have not been available. Treatment with the natural ligand angiotensin II primarily elicits effects mediated by the dominating AT₁R; the only available AT₂R antagonist, PD123319, is nonselective at higher doses²; and the only available AT₁ agonist, CGP42112, is a peptide and furthermore has agonistic and

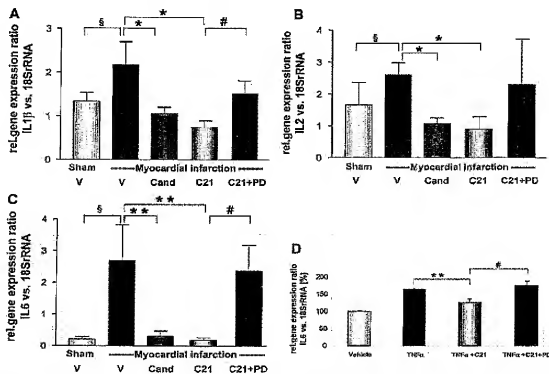


Figure 6. Expression of IL-1 β (A), IL-2 (B), and IL-6 (C) in the peri-infarct zone in rat hearts. MI caused a significant increase in the expression of all cytokines examined (sham vs MI/vehicle [V]: § P <0.05, §§ P <0.01; n =6 per group). Elevated expression levels were significantly reduced either by candesartan (cand; 0.1 mg/kg per day IP) or by C21 (0.03 mg/kg per day IP) (vehicle vs Cand: § P <0.05, §§ P <0.01; vehicle vs C21: § P <0.05, §§ P <0.01; n =6 per group). The effect of C21 was inhibited by cotreatment with PD123319 (PD; 3 mg/kg per day IP) (C21 vs C21+PD: §§ P <0.05, §§ P <0.01; n =6 per group). D, IL-6 expression in H9C2 neonatal rat cardiomyocytes. TNF- α -induced (10 ng/mL) IL-6 expression was significantly reduced by C21 (10⁻⁶ mol/L). The effect of C21 was abolished by cotreatment with PD123319 (PD; 10⁻⁶ mol/L) († of 3 independent experiments; n =3 per group; §§ P <0.01, § P =0.01). rel. indicates relative.

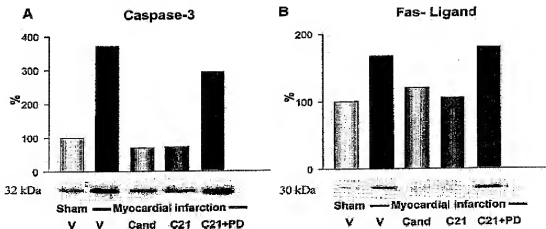


Figure 7. Western blot analysis of caspase-3 (A) and Fas-ligand (B) expression in the peri-infarct zone of rat hearts. Shown is 1 representative of 3 blots for each marker. Note the increase in caspase-3 and Fas-ligand expression after MI, which was suppressed by candesartan (Cand; 0.1 mg/kg per day IP) or C21 (0.03 mg/kg per day IP). Cotreatment with PD123319 (PD; 3 mg/kg per day) blocked the effect of C21. V indicates vehicle.

antagonistic properties, which renders the interpretation of *in vivo* data difficult.

We present the first *in vivo* study using a specific and selective nonpeptide AT₂R agonist, C21.⁷ MI was induced in normotensive Wistar rats by permanent ligation of the left anterior descending coronary artery. Subsequently, animals were treated with C21 for 1 week. Direct AT₂R stimulation by C21 improved systolic and diastolic cardiac function after MI, coinciding with a smaller infarct scar in C21-treated animals compared with vehicle treatment. Whereas MI led to an impairment of all parameters measured by echocardiography (LVIDs, EF, fractional shortening for systolic function; LVIDd, E, A, E/A, EDT for diastolic function) or Millar catheter (contractility, dP/dt_{max} for systolic function; LVEDP, dP/dt_{min} for diastolic function), respectively, treatment with C21 improved all of these parameters significantly without

exception. For most parameters, the effect of C21 could be inhibited by cotreatment with the established AT₂R antagonist PD123319, thus providing good evidence that the effects seen by C21 treatment are indeed a result of specific AT₂R stimulation.

Previous studies addressing the role of the AT₂R in cardiac function and remodeling after MI were performed primarily in genetically altered mice either overexpressing or lacking AT₂Rs.⁸ The vast majority of these studies report a beneficial effect of the presence of AT₂Rs on cardiac function after MI. For instance, Bove et al and Yang et al observed improved postinfarct cardiac function in AT₂R-overexpressing mice, with NO apparently being of pivotal importance.^{9–11} Several other studies used male AT₂R^{-/-} mice and reported a deteriorated outcome after MI compared with wild-type mice.^{12–15} Xu et al¹⁶ did not detect any differences between AT₂R^{-/-} and AT₂R^{+/+} mice 4 weeks after MI but detected an impaired response to AT₁R blockers in AT₂R^{-/-} mice. Ichihara et al¹⁷ used male AT₂R^{-/-} mice as well, but, in contrast to the other studies using this model, postinfarct scar area was reduced in knockout mice. Nevertheless, mortality in AT₂R^{-/-} mice was higher than in wild-type mice as a result of an increased rate of cardiac ruptures within the first week after ligation. Between weeks 2 and 6 after MI, mortality rate was identical in AT₂R^{-/-} and wild-type mice.

Our study differs from those discussed above because these studies examined effects related to AT₂R expression and not to specific receptor stimulation. Admittedly, there may be some endogenous postinfarct AT₂R stimulation by elevated cardiac angiotensin II levels. Furthermore, the AT₂R has been reported to possess constitutive intrinsic activity,¹⁸ and cardiac AT₂R expression is increased after MI.^{19,20} Still, the intensity of such an endogenous stimulation is very unlikely to reach the intensity achieved by stimulation with a specific agonist at an appropriate, pharmacological dosage.

Apart from rats treated with different doses of C21, our study also included a group of animals treated with the AT₁R blocker candesartan. We used an AT₁R blocker as a reference drug (1) because of its proven efficacy in the improvement of postinfarct cardiac function as shown in animals and clinical

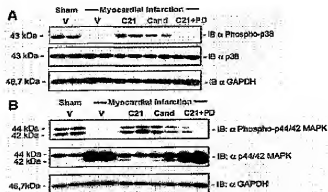


Figure 8. Western blot analysis of phospho-p38 MAPK (A) and phospho-p44/42 MAPK (B) expression in the peri-infarct zone of rat hearts. Shown is 1 representative of 3 blots for each marker. Note the complete loss of phospho-p38 and phospho-p44/42 MAPK expression after MI. Phospho-p38 and phospho-p44/42 MAPK expression was only partly restored by candesartan (Cand; 0.1 mg/kg per day IP), whereas C21 (0.03 mg/kg per day IP) led to an almost complete rescue of MAPK expression. Cotreatment with PD123319 (PD; 3 mg/kg per day) blocked the effect of C21. V indicates vehicle.

cally⁴ and (2) because it may interfere with the renin-angiotensin system at least in part by indirect AT₁R stimulation. We did not intend to compare the efficacy but rather the quality of actions of both drugs because, at present, with only very limited data and experimental experience with C21 available, it is hard to define what an equally effective dose of C21 or candesartan would be. Nevertheless, in mild to moderate infarctions, both drugs restored a number of cardiac parameters back to levels in noninfarcted animals (ie, with regard to these parameters, both drugs elicited an equal maximal response) (E, A, E/A, EDT). In regard to those parameters, in which a maximal response was not achieved, thus allowing a "comparison" of C21 and candesartan actions (LVIDs, LVIDd, EDP, contractility, dP/dt_{max}), the improvement by C21 was more effective than with candesartan. This outcome applied to systolic and diastolic function and may point to a superiority of C21.

Outcome of animals treated with the combination of C21 plus candesartan was not superior to that of animals treated with C21 alone, as may have been expected. A likely reason for this finding could be that C21 monotherapy already elicited a maximal functional improvement, which did not admit any further improvement.

To examine the molecular mechanisms underlying the beneficial effect of C21 on post-MI cardiac function, we looked for a putative effect of this drug on the apoptotic and inflammatory response in the peri-infarct zone.

MI led to a significant increase in Fas-ligand and caspase-3 expression in the infarct border zone. C21 significantly reduced the elevated, MI-related Fas-ligand and caspase-3 expression, and this effect could be blocked by the specific AT₂R antagonist PD123319. Because apoptosis is a major contributor in postinfarct cardiac remodeling, including the development of infarct expansion,²¹ prevention of cardiomyocyte apoptosis by C21 may be a major molecular mechanism by which AT₂R stimulation preserves cardiac function after MI.

The MAPKs are involved in numerous cellular processes such as apoptosis, inflammation, proliferation, or hypertrophy.

In our study, we found that the activity of p38 MAPK and p44/42 MAPK was reduced to undetectable levels 7 days after MI in vehicle-treated animals and that treatment with C21 was able to completely rescue p38 MAPK and p44/42 MAPK expression in infarcted hearts. This effect was blocked by PD123319, indicating again AT₂R specificity of the C21 effect. The effect of candesartan was by far weaker than the effect of C21.

Although MAPKs have traditionally been regarded as mediators of such processes as proliferation, apoptosis, and inflammation, recent data provided evidence that MAPK activation can also result in beneficial effects directly opposed to the aforementioned effects, namely, antiproliferation, cell survival, and anti-inflammation.²² In our experimental setting, the rescue of p38 MAPK and p44/42 MAPK expression by C21 7 days after MI coincided with an improvement of cardiac function and a smaller scar size. Although this is an observation of coincidence, a causal relationship between MAPK activation and improved cardiac function is supported by a recent study by Tenhunen et al,²³ in which the authors were able to improve postinfarct cardiac

function by rescuing p38 MAPK expression through local adenovirus-mediated p38 MAPK gene transfer. Functionally, MAPK rescue reduced fibrosis and apoptosis (the latter shown by terminal deoxynucleotidyl transferase dUTP nick end labeling staining and, as in our study, by a reduction of caspase-3). Thus, the increase in p38 and p44/42 MAPK expression by C21 may be part of its antiapoptotic effect after MI, which is also reflected by a decrease in caspase-3 and Fas ligand.

In the early phase after acute MI, necrosis of cardiac cells sets into motion an inflammatory response that ultimately promotes fibrosis, scar formation, and thereby heart failure. C21 suppressed this postinfarct inflammatory response, as shown by a significant decrease in cytokine (MCP-1, IL-1 β , IL-2, IL-6) expression in plasma and the peri-infarct zone. Again, the effect of C21 could be blocked by the AT₂R antagonist, indicating the AT₂R specificity of this effect. Because it has been shown for other drugs that inhibition of the inflammatory response contributes to the preservation of cardiac function, it can be assumed that anti-inflammation may be another mechanism of action of AT₂R stimulation by C21.

It must be pointed out that this study was performed in normotensive rats and that no changes in blood pressure or heart rate occurred over the time of the experiment either by C21 at all doses used or by candesartan, which was given in a non-blood pressure-lowering dose. Therefore, the effects on cardiac function and cytokine and apoptosis markers as well as MAPK expression are unlikely to be caused by treatment-related hemodynamic changes. This notion is further supported by the inhibitory effect of C21 on IL-6 expression in rat fetal cardiomyocytes *in vitro* (ie, in an experimental setting independent of any hemodynamic influences).

In sum, this is the first study to use a nonpeptide AT₂R agonist for direct stimulation of the AT₂R after MI in rats. Treatment with C21 over a period of 7 days led to a pronounced improvement of systolic and diastolic cardiac function coinciding with a smaller scar volume. Furthermore, C21 reduced apoptosis and acted in an anti-inflammatory manner, suggesting that these mechanisms are contributing to the beneficial effect of AT₂R stimulation. AT₂R stimulation may thus represent a potential future therapeutic option for the improvement of postinfarct cardiac function but also for the prevention of end-organ damage in further organ systems.

Limitations

Because in our study infarct size has only been measured at the end but not before treatment, we cannot exclude an unmeasured difference in infarct size between groups at baseline, which may have influenced the outcome of the study. However, this possibility has been minimized by grouping of animals according to EF before the start of treatment to keep differences in mean EF between groups minimal. This procedure is based on the observation by us and others that EF correlated well with infarct size ($r^2=0.671$; $P<0.0001$).²⁴ Moreover, assignment of animals to the respective treatment groups was performed by an investigator who was blinded to the outcome of EF measurement in individual animals (only knowing whether EF was $>35\%$ or $\leq 35\%$).

Sources of Funding

Dr Grzesiak and C. Curato received a grant from the EU Marie Curie graduate program CARDIOVASC (MEST-CT-2005-020268).

Disclosures

Drs Dahlöf and Unger have modest ownership interest in Vicore Pharma and received speaker fees. Dr Stockelings received speaker fees from Vicore Pharma. The other authors report no conflicts.

References

- Stoll M, Stockelings UM, Paul M, Bortolotti SP, Metzger R, Unger T. The angiotensin AT₂-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest*. 1995;95:651–657.
- Nakajima M, Hutchinson HG, Fujikawa M, Hayashida W, Morizata R, Zhang L, Horiochi M, Pratt RE, Dora VI. The angiotensin II type 2 (AT₂) receptor antagonizes the growth effects of the AT₁ receptor: gene-of-function study using gene transfer. *Proc Natl Acad Sci U S A*. 1995;92:10663–10667.
- Stockelings UM, Kaschinske E, Unger T. The AT₂-receptor: a matter of love and hate. *Peptides*. 2005;26:1401–1409.
- Pfeiffer MA, McIlrath JIV, Velazquez EJ, Rouleau JL, Kober L, Maggioni AP, Solomon SD, Swedberg K, Van de Werf F, White H, Leimberger JD, Herris M, Edwards S, Zeinikofsky S, Sellers MA, Califf RM. Valsartan, captopril, or both in myocardial infarction complicated by heart failure, left ventricular dysfunction, or both. *N Engl J Med*. 2003; 349:1893–1906.
- Widdop RE, Jones ES, Hinnin RL, Gaspari TA. Angiotensin AT₂ receptors: cardiovascular hope or hype? *Circulation*. 2002;106:829–834.
- Macard D, Bortolotti SP, Whitehead S, De Gasparo M, Levens N. Renal actions of the selective angiotensin AT₂ receptor ligands CGP 42112B and PD 123191 in the sodium-depleted rat. *Eur J Pharmacol*. 1993;249:85–93.
- Wan Y, Wallinder C, Plouffe B, Beaucy H, Mahalingam AK, Wu X, Johansson B, Holm M, Bortolotti M, Karlén A, Pentzén A, Nyberg F, Fändriks L, Gallo-Payot N, Hallberg A, Altman M. Design, synthesis, and biological evaluation of the first selective nonpeptide AT₂ receptor agonist. *J Med Chem*. 2004;47:5993–6006.
- Litwin SE, Katz SE, Morgan JP, Douglas PS. Serial echocardiographic assessment of left ventricular geometry and function after large myocardial infarction in the rat. *Circulation*. 1994;89:345–354.
- Bove CM, Yang Z, Gilson WD, Epstein FH, French BA, Berr SS, Bishop SP, Matsubara H, Carey RH, Kramer CM. Nitric oxide mediates benefits of angiotensin II type 2 receptor overexpression during post-infarct remodeling. *Hypertension*. 2004;43:680–685.
- Bove CM, Gilson WD, Scott DC, Epstein FH, Yang Z, Dimaria JM, Berr SS, French BA, Bishop SP, Kramer CM. The angiotensin II type 2 receptor and improved adjacent region function post-MI. *J Cardiovasc Magn Reson*. 2005;7:459–464.
- Yang Z, Bove CM, French BA, Epstein FH, Berr SS, DiMaria JM, Gilson JJ, Carey RM, Kramer CM. Angiotensin II type 2 receptor overexpression preserves left ventricular function after myocardial infarction. *Circulation*. 2002;106:105–111.
- Adachi Y, Saito Y, Kishimoto I, Harada M, Kuwahara K, Takahashi N, Kawakami R, Nakamishi M, Nakagawa Y, Tanimoto K, Saitoh Y, Yasuno S, Usami S, Iwan M, Horiochi M, Kikura K. Angiotensin II type 2 receptor deficiency exacerbates heart failure and reduces survival after acute myocardial infarction in mice. *Circulation*. 2003;107:2406–2408.
- Oishi Y, Ozono R, Yano Y, Teramishi Y, Akishita M, Horiochi M, Oshima T, Kanbe M. Cardioprotective role of AT₂ receptor in postinfarction left ventricular remodeling. *Hypertension*. 2003;41:814–818.
- Oishi Y, Ozono R, Yoshizumi M, Akishita M, Horiochi M, Oshima T. AT₂ receptor mediates the cardioprotective effects of AT₁ receptor antagonist in post-myocardial infarction remodeling. *Life Sci*. 2006;80:872–88.
- Vargas S, Yang Z, Bove CM, Gilson WD, Epstein FH, French BA, Berr SS, Bishop SP, Conaway MR, Matsubara H, Carey RM, Kramer CM. Interaction between AT₁ and AT₂ receptors during postinfarction left ventricular remodeling. *Am J Physiol*. 2006;290:H1004–H1010.
- Xu J, Carretero OA, Liu YH, Shesely EG, Yang F, Kapke A, Yang XP. Role of AT₂ receptors in the cardioprotective effect of AT₁ antagonists in mice. *Hypertension*. 2002;40:244–250.
- Ishihara S, Senbonmatsu T, Price EJ, Ishihara T, Gaffney FA, Inagami T. Targeted deletion of angiotensin II type 2 receptor causes cardiac rupture after acute myocardial infarction. *Circulation*. 2002;106:2244–2249.
- Miera S, Karnik SS, Saku K. Constitutively active homo-oligomeric angiotensin II type 2 receptor induces cell signaling independent of receptor conformation and ligand stimulation. *J Biol Chem*. 2005;280: 18237–18244.
- Nio Y, Matsubara H, Murasawa S, Kanazaki M, Inada M. Regulation of gene transcription of angiotensin II receptor subtypes in myocardial infarction. *J Clin Invest*. 1995;95:46–54.
- Volpe M, Musumeci B, de Paolis P, Savola C, Morganti A. Angiotensin II AT₂ receptor subtype: an upraising frontier in cardiovascular disease? *J Hypertens*. 2003;21:1429–1443.
- Diwan A, Krusz M, Syed FM, Wansapura J, Ren X, Koesters AG, Li H, Kirshenbaum LA, Hahn HS, Robbins J, Jones WK, Dorn GW II. Inhibition of ischemic cardiomyocyte apoptosis through targeted inhibition of Bnip3 restrains postinfarction remodeling in mice. *J Clin Invest*. 2007;117: 2825–2833.
- Lips DJ, Bueno OF, Wilkins BJ, Purcell NH, Kaiser RA, Lorenz JN, Volin L, Saba-El-Leil MK, Meloche S, Pouyssegur J, Pagès G, De Wintt LJ, Doevendans PA, Molkentin JD. MEK1-ERK1 signaling pathway protects myocardium from ischemic injury in vivo. *Circulation*. 2004;109: 1938–1941.
- Tenbaun O, Saini Y, Ilves M, Rysa J, Tuukkanen J, Sepi R, Pennanen H, Ruskola H, Leskinen H. p38 Kinase rescues failing myocardium after myocardial infarction: evidence for angiogenic and antiapoptotic mechanisms. *FASEB J*. 2006;20:E1276–E1286.
- Sciagra R, Imperiale A, Antonucci D, Miglicioni A, Parodi G, Comis G, and Pupi A. Relationship of infarct size and severity versus left ventricular ejection fraction and volumes obtained from 99mTc-sestamibi gated single-photon emission computed tomography in patients treated with primary percutaneous coronary intervention. *Eur J Nucl Med Mol Imaging*. 2004;31:969–974.

CLINICAL PERSPECTIVE

Interference with the renin-angiotensin system is a very common, well-established, and successful concept of treatment in cardiovascular disease. All currently available and approved renin-angiotensin system–interfering drugs (angiotensin-converting enzyme [ACE] inhibitors, angiotensin II type 1 [AT₁] receptor [AT₁R] blockers, renin inhibitors, aldosterone antagonists) aim at inhibition of the unfavorable effects of an overactivated renin-angiotensin system signaling via the AT₁R. However, the renin-angiotensin system not only harbors the renin/ACE/AT₁-aldosterone axis, which, when overstimulated, is involved in a huge variety of pathologies ranging from hypertension to end-organ damage or local processes in myocardial infarction and stroke; in fact, it also possesses a tissue-protective axis involving ACE₂, Ang-(1–7), and the AT₂ receptor (AT₂R), which seems to counteract detrimental effects mediated via the AT₁R but also inflammation, hyperproliferation, or fibrosis in general. At present, no pharmacological tool is available to specifically stimulate this beneficial ACE₂/Ang-(1–7)/AT₂R axis in vivo. The novel nonpeptide AT₂R agonist compound 21 may be such a drug. The study presented here is the first to test the therapeutic potential of direct AT₂R stimulation in a model of myocardial infarction. Compound 21 significantly improved cardiac function and resulted in a smaller scar size independent of any hemodynamic changes. Because compound 21 is a nonpeptide with a sufficient bioavailability (~30%), it may be a suitable lead compound for the development of a drug for the blood pressure-independent treatment or prevention of end-organ damage in humans.

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT C

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
5 December 2002 (05.12.2002)

PCT

(10) International Publication Number
WO 02/096883 A1(51) International Patent Classification: C07D 233/54,
A61K 31/4174, A61P 43/00, G01N 33/00, C07D 409/10Department of Medicinal Chemistry, Biomedical Centre,
Uppsala University, P.O. Box 574, S-751 23 Uppsala (SE).

(21) International Application Number: PCT/GB02/02563

(74) Agent: MCNEENEY, Stephen, P.; Eric Potter Clarkson,
Park View House, 58 The Ropewalk, Nottingham NG1
5DD (GB).

(22) International Filing Date: 30 May 2002 (30.05.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0113129.1 31 May 2001 (31.05.2001) GB
0121511.8 7 September 2001 (07.09.2001) GB
60/350,959 25 January 2002 (25.01.2002) US
0201794.5 26 January 2002 (26.01.2002) GB(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU,
ZA, ZW.(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Burasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,
NE, SN, TD, TG).(71) Applicant (*for all designated States except US, UZ*): VI-
CORE PHARMA AB [SE/SE]; Uppsala Science Park,
S-751 83 Uppsala (SE).(71) Applicant (*for UZ only*): MCNEENEY, Stephen, Phillip
[GB/GB]; Park View House, 58 The Ropewalk, Notting-
ham NG1 5DD (GB).

(72) Inventors; and

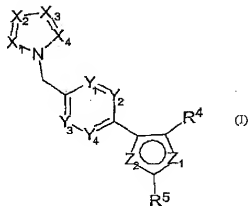
(75) Inventors/Applicants (*for US only*): HALLBERG,
Anders [SE/SE]; Department of Medicinal Chemistry,
Biomedical Centre, Uppsala University, P.O. Box 574,
S-751 23 Uppsala (SE); ALTERMAN, Mathias [SE/SE];

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: TRICYCLIC COMPOUNDS USEFUL AS ANGIOTENSIN II AGONISTS



(57) Abstract: There is provided compounds of formula (I), wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 , Z_3 , Z_4 , Z_5 , R^4 and R^5 have meanings given in the description, and pharmaceutically-acceptable salts thereof, which compounds are useful as selective agonists of the AT₂ receptor, and thus, in particular, in the treatment of *inter alia* gastrointestinal conditions, such as dyspepsia, IBS and MOF, and cardiovascular disorders.

TRICYCLIC COMPOUNDS USEFUL AS ANGIOTENSIN II AGONISTS

Field of the Invention

This invention relates to novel pharmaceutically-useful compounds, in particular compounds that are angiotensin II (AngII) agonists, more particularly agonists of the AngII type 2 receptor (hereinafter the AT2 receptor), and especially agonists that bind selectively to that receptor. The invention further relates to the use of such compounds as medicaments, to pharmaceutical compositions containing them, and to synthetic routes to their production.

Background and Prior Art

The endogenous hormone AngII is a linear octapeptide (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸), and is the active component of the renin-angiotensin system (RAS). It is produced by the sequential processing of the pro-hormone angiotensinogen by renin and angiotensin converting enzyme (ACE).

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure, body fluid and electrolyte homeostasis. Ang II exerts these physiological actions in many organs including the kidneys, the adrenal glands, the heart, blood vessels, the brain, the gastrointestinal tract and the reproductive organs (de Gasparo *et al*, *Pharmacol. Rev.* (2000) 52, 415-472).

Two main classes of AngII receptors have been identified, and designated as the type 1 receptor (hereinafter the AT1 receptor) and the AT2 receptor.

The AT1 receptor is expressed in most organs, and is believed to be responsible for the majority of the biological effects of AngII. The AT2 receptor is more prevalent than the AT1 receptor in fetal tissues, the adult ovaries, the adrenal medulla and the pancreas. An equal distribution is reported in the brain and uterus (Ardailiou, *J. Am. Soc. Nephrol.*, **10**, S30-39 (1999)).

Several studies in adult individuals appear to demonstrate that, in the modulation of the response following AngII stimulation, activation of the AT2 receptor has opposing effects to those mediated by the AT1 receptor.

The AT2 receptor has also been shown to be involved in apoptosis and inhibition of cell proliferation (see de Gasparo *et al, supra*). Further, it seems to play a role in blood pressure control. For example, it has been shown in transgenic mice lacking AT2 receptors that their blood pressure was elevated. Furthermore, it has been concluded that the AT2 receptor is involved in exploratory behaviour, pain sensitivity and thermoregulation.

The expression of AT2 receptors has also been shown to increase during pathological circumstances, such as vascular injury, wound healing and heart failure (see de Gasparo *et al, supra*).

The expected pharmacological effects of agonism of the AT2 receptor are described generally in de Gasparo *et al, supra*.

More recently, AT2 receptor agonists have been shown to be of potential utility in the treatment and/or prophylaxis of disorders of the alimentary tract, such as dyspepsia and irritable bowel syndrome, as well as multiple organ failure (see international patent application WO 99/43339).

AngII antagonists (which bind to the AT1 and/or AT2 receptors) have been disclosed in *inter alia* European patent applications EP 409 332, EP 512 675; international patent applications WO 94/27597, WO 94/02142, WO 95/23792 and WO 94/03435; and US patent numbers 5,091,390, 5,177,074, 5,412,097, 5,520,521, 5,260,285, 5,376,666, 5,252,574, 5,312,820, 5,330,987, 5,166,206, 5,932,575 and 5,240,928. AngII agonists, and particularly AT2 receptor agonists, are not contemplated in any of these documents.

- 10 International patent application WO 00/68226 and US patent number 6,235,766 disclose compounds comprising substituted imidazolyl groups, which groups are attached, *via* a methylene bridge, to a phenylthiophene moiety, as agonists of angiotensin-(1-7) receptors. International patent application WO 01/44239 discloses biphenylsulfonamide compounds as
15 combined angiotensin and endothelin receptor antagonists. The use of the compounds as Ang II receptor agonists is neither mentioned nor suggested in any of these documents.

- US patent number 5,444,067 discloses compounds comprising a 5,7-
20 dimethyl-2-ethylpyridinoimidazolyl group attached, *via* a methylene bridge, to a phenylthiophene moiety, as AT2 receptor agonists. The use of unsubstituted imidazole-containing compounds is neither mentioned nor suggested.

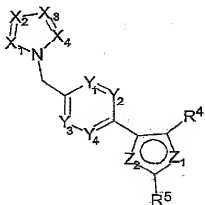
- 25 Peptide and non-peptide AT2 receptor agonists, unrelated structurally to those described herein, and potential uses thereof, have been disclosed in, for example, international patent applications WO 00/38676, WO 00/56345, WO 00/09144, WO 99/58140, WO 99/52540, WO 99/46285, WO 99/45945, WO 99/42122, WO 99/40107, WO 99/40106, WO 99/39743,

WO 99/26644, WO 98/33813, WO 00/02905 and WO 99/46285; US patent number 5,834,432; and Japanese patent application JP 143695.

- However, there remains a need for effective and/or selective AT₂ receptor agonists, which are expected to find utility in *inter alia* the above-mentioned conditions.

Disclosure of the Invention

- According to the invention there is provided a compound of formula I,



- wherein
- one of X_1 and X_2 represents $-N-$ and the other represents $-C(R^1)-$;
 - X_3 represents $-N-$ or $-C(R^2)-$;
 - X_4 represents $-N-$ or $-C(R^3)-$;
 - R^1 , R^2 and R^3 independently represent H, C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkoxy- C_{1-6} -alkyl or halo;
 - provided that, when X_1 represents $-C(R^1)-$, X_3 represents $-C(R^2)-$ and X_4 represents $-C(R^3)-$, then R^1 represents H;
 - Y_1 , Y_2 , Y_3 and Y_4 independently represent $-CH-$ or $-CF-$;
 - Z_1 represents $-CH-$, $-O-$, $-S-$, $-N-$ or $-CH=CH-$;

Z_2 represents $-\text{CH}-$, $-\text{O}-$, $-\text{S}-$ or $-\text{N}-$;

provided that:

- (a) Z_1 and Z_2 are not the same;
- (b) when Z_1 represents $-\text{CH}=\text{CH}-$, then Z_2 may only represent $-\text{CH}-$ or $-\text{N}-$; and
- (c) other than in the specific case in which Z_1 represents $-\text{CH}=\text{CH}-$, and Z_2 represents $-\text{CH}-$, when one Z_1 and Z_2 represents $-\text{CH}-$, then the other represents $-\text{O}-$ or $-\text{S}-$;

R^4 represents $-\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^6$, $-\text{S}(\text{O})_2\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^6$, $-\text{C}(\text{O})\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^6$,
or, when Z_1 represents $-\text{CH}=\text{CH}-$, R^4 may represent
 $-\text{N}(\text{H})\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^7$ or $-\text{N}(\text{H})\text{C}(\text{O})\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^7$;

R^5 represents C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkoxy- C_{1-6} -alkyl or di- C_{1-3} -alkylamino- C_{1-4} -alkyl;

R^6 represents C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkoxy- C_{1-6} -alkyl,
 C_{1-3} alkoxy- C_{1-6} -alkoxy, C_{1-6} alkylamino or di- C_{1-6} alkylamino; and
 R^7 represents C_{1-6} alkyl,

or a pharmaceutically-acceptable salt thereof,

which compounds and salts are referred to together hereinafter as "the compounds of the invention".

20

Pharmaceutically-acceptable salts include acid addition salts and base addition salts. Such salts may be formed by conventional means, for example by reaction of a free acid or a free base form of a compound of the invention with one or more equivalents of an appropriate acid or base,
optionally in a solvent, or in a medium in which the salt is insoluble,
followed by removal of said solvent, or said medium, using standard techniques (e.g. *in vacuo* or by freeze-drying). Salts may also be prepared by exchanging a counter-ion of a compound of the invention in the form of a salt with another counter-ion, for example using a suitable ion exchange resin.

30

Unless otherwise specified, alkyl groups, and the alkyl parts of alkoxy, alkoxyalkyl, alkoxyalkoxy, alkylamino and alkylaminoalkyl groups, as defined herein may be straight-chain or, when there is a sufficient number
5 (i.e. a minimum of three) of carbon atoms, be branched-chain, and/or cyclic. Further, when there is a sufficient number (i.e. a minimum of four) of carbon atoms, such groups may also be part cyclic/acyclic. Such alkyl groups, and alkyl parts of alkoxy, alkoxyalkyl, alkoxyalkoxy, alkylamino and alkylaminoalkyl groups, may also be saturated or, when there is a
10 sufficient number (i.e. a minimum of two) of carbon atoms, be unsaturated. Unless otherwise specified, such groups may also be substituted by one or more halo, and especially fluoro, atoms.

For the avoidance of doubt, alkoxy and alkoxyalkoxy groups are attached to
15 the rest of the molecule *via* the oxygen atom in that group, alkylamino groups are attached to the rest of the molecule *via* the nitrogen atom of the amino part of that group and alkylaminoalkyl and alkoxyalkyl groups are attached to the rest of the molecule *via* the alkyl part of that group.

20 The term "halo", when used herein, includes fluoro, chloro, bromo and iodo.

Preferred ring systems comprising the substituents Y_1 , Y_2 , Y_3 and Y_4 include phenyl groups. For the avoidance of doubt, the ring systems in compounds of formula I that comprise the groups Z_1 and Z_2 , are aromatic in
25 nature. In some instances, for example in cases where one or more of Z_1 and Z_2 represent $-CH-$ or $-N-$ the skilled person will appreciate that an additional H atom may necessarily be bonded to that CH group or N atom, in order to ensure that the rules of valency are adhered to. Preferred ring systems comprising Z_1 and Z_2 include oxazole groups, thiazole groups,
30 phenyl groups, pyridinyl groups, thiophenyl groups and furanyl groups.

In this respect, compounds of the invention may exhibit tautomerism. All tautomeric forms and mixtures thereof are included within the scope of the invention.

5 Compounds of the invention also contain one or more asymmetric carbon atoms and may therefore exhibit optical and/or diastereoisomerism. Diastereoisomers may be separated using conventional techniques, e.g. chromatography or fractional crystallisation. The various stereoisomers
10 may be isolated by separation of a racemic or other mixture of the compounds using conventional, e.g. fractional crystallisation or HPLC, techniques. Alternatively the desired optical isomers may be made by reaction of the appropriate optically active starting materials under conditions which will not cause racemisation or epimerisation, or by
15 derivatisation, for example with a homochiral acid followed by separation of the diastereomeric derivatives by conventional means (e.g. HPLC, chromatography over silica). All stereoisomers are included within the scope of the invention.

20 Preferred compounds of the invention include those in which:

- (i) when X_1 represents $-C(R^1)-$, then:
 - (a) X_3 represents $-C(R^3)-$ and X_4 represents $-N-$;
 - (b) X_3 and X_4 both represent N ; or
 - (c) X_3 represents $-C(R^3)-$ and X_4 represents $-C(R^3)-$; or
- 25 (ii) when X_1 represents $-N-$, then
 - (a) X_3 represents $-N-$; or
 - (b) X_3 represents $-C(R^3)-$ and X_4 represents $-C(R^3)-$.

In case (i)(a) above, it is further preferred that R^1 represents H.

In case (ii)(a) above, when X_4 represents $-C(R^3)-$, it is further preferred that R^3 represents H.

Preferred compounds of formula I include those in which:

- 5 R^1 represents C_{1-3} alkyl, such as ethyl, $-CF_3$ or, especially, H;
 R^2 represents C_{1-3} alkyl, such as methyl, halo, or, especially, H;
 R^3 represents C_{1-3} alkyl, halo or, especially, H;
 Y_1, Y_2, Y_3 and Y_4 all represent $-CH-$;
 Z_1 represents $-S-$ or $-CH=CH-$;
10 Z_2 represents $-CH-$;
 R^4 represents $S(O)_2N(H)C(O)R^6$;
 R^5 represents *n*-butyl or, particularly, *iso*-butyl;
 R^6 represents *n*-butoxymethyl, *iso*-butoxy and especially, *n*-butoxy.
- 15 Preferred ring systems comprising the substituents X_1, X_2, X_3 and X_4 include pyrazole groups, imidazole groups, 1,2,4-triazole groups and tetrazole groups.

- Compounds of the invention that may be mentioned include those in which,
20 when X_1, X_3 and X_4 all represent $-CH-$, Y_1, Y_2, Y_3 and Y_4 all represent $-CH-$, Z_1 represents $-CH=CH-$ or, particularly, $-S-$, Z_2 represents $-CH-$ and R^5 represents *n*-butyl or, particularly, *iso*-butyl, then R^4 represents $-S(O)_2N(H)C(O)R^6$, in which R^6 represents $-O$ -*iso*-propyl (i.e. *iso*-propoxy), $-O$ -*iso*-butyl (i.e. *iso*-butoxy), $-CH_2-O$ -*n*-butyl (i.e. *n*-butoxymethyl) or,
25 particularly, $-O$ -*n*-butyl (i.e. *n*-butoxy).

- Compounds of the invention that may further be mentioned include those in which, when X_1, X_3 and X_4 all represent $-CH-$, Y_1, Y_2, Y_3 and Y_4 all represent $-CH-$, Z_1 represents $-CH=CH-$ or $-S-$, Z_2 represents $-CH-$ and R^5
30 represents *n*-butyl or *iso*-butyl, then R^4 does not represent

$-S(O)_2N(H)C(O)R^6$, in which R^6 represents $-O$ -*iso*-propyl, $-O$ -*iso*-butyl, $-CH_2-O$ -*n*-butyl or $-O$ -*n*-butyl.

Further compounds of the invention that may be mentioned include those in which:

R^4 does not represent $-S(O)_2N(H)S(O)_2R^6$;

R^5 does not represent di- C_{1-3} alkylamino- C_{1-4} -alkyl;

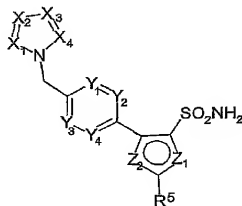
R^6 does not represent C_{1-3} alkoxy- C_{1-6} alkoxy.

More preferred compounds of the invention include the compounds of the examples described hereinafter.

Compounds of formula I may be made in accordance with techniques well known to those skilled in the art, for example as described hereinafter.

According to a further aspect of the invention there is provided a process for the preparation of a compound of formula I, which process comprises:

(i) for compounds of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ or $-S(O)_2N(H)S(O)_2R^6$, and R^6 is as hereinbefore defined, reaction of a compound of formula II,



II

wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as hereinbefore defined with a compound of formula III,

5



III

wherein G represents C(O) or S(O)₂ (as appropriate), L¹ represents a suitable leaving group, such as halo (e.g. chloro or bromo) and R⁶ is as hereinbefore defined, for example at around room temperature or above
10 (e.g. up to 60-70°C) in the presence of a suitable base (e.g. pyrrolidinopyridine, pyridine, triethylamine, tributylamine, trimethylamine, dimethylaminopyridine, di-*iso*-propylamine, 1,8-diazabicyclo[5.4.0]undec-7-ene, sodium hydroxide, or mixtures thereof) and an appropriate solvent (e.g. pyridine, dichloromethane, chloroform, tetrahydrofuran,
15 dimethylformamide, trifluoromethylbenzene or triethylamine). Preferred base/solvent systems for compounds of formula III in which G is C(O) include pyrrolidinopyridine/pyridine, pyrrolidinopyridine/triethylamine, dimethylaminopyridine/pyridine or dimethylaminopyridine/triethylamine. Preferred base/solvent systems for compounds of formula III in which G is
20 S(O)₂ include NaOH/THF;

(ii) for compounds of formula I in which R⁴ represents -S(O)₂N(H)C(O)R⁶ and R⁶ represents C₁₋₆ alkoxy-C₁₋₆-alkyl, coupling of a compound of formula II as hereinbefore defined with a compound of formula IV,

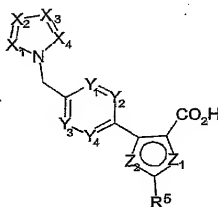
25



IV

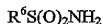
wherein R^{6a} represents C₁₋₆ alkoxy-C₁₋₆-alkyl, for example under similar conditions to those described under process step (i) above, in the presence
30 of a suitable coupling reagent (e.g. 1,1'-carbonyl-diimidazole, N,N'-

- dicyclohexylcarbodiimide, N,N' -disuccinimidyl carbonate, benzotriazole-1-
 yloxytris(dimethylamino)phosphoniumhexafluorophosphate, 2-(1H-
 benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate,
 benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate,
 5 bromo-tris-pyrrolidinophosphonium hexafluorophosphate or 2-(1H-
 benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluorocarbonate), a
 suitable base (as mentioned in process step (i) above) and an appropriate
 solvent (as mentioned in process step (i) above);
- 10 (iii) for compounds of formula I in which R^4 represents $-C(O)N(H)S(O)_2R^6$
 and R^6 is as hereinbefore defined, coupling of a compound of formula V,



V

- 15 wherein $X_1, X_2, X_3, X_4, Y_1, Y_2, Y_3, Y_4, Z_1, Z_2$ and R^5 are as hereinbefore
 defined with a compound of formula VI,



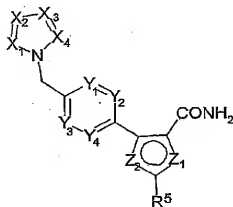
VI

- 20 wherein R^6 is as hereinbefore defined, for example in the presence of a
 suitable coupling reagent (such as those described in process step (ii)
 hereinbefore), and under similar reaction conditions to those described

12

hereinbefore for preparation of compounds of formula I in which R^6 represents C_{1-6} alkoxy- C_{1-6} -alkyl;

- (iv) for compounds of formula I in which R^4 represents $-C(O)N(H)S(O)_2R^6$
 5 and R^5 is as hereinbefore defined, coupling of a compound of formula VII,



VII

- wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as hereinbefore
 10 defined with a compound of formula VIII,

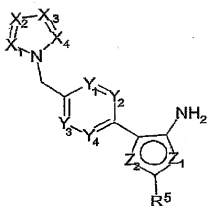


VIII

- wherein R^6 is as hereinbefore defined, for example at around $50^\circ C$ in the
 presence of a suitable base (e.g. sodium hydride) and an appropriate organic
 15 solvent (e.g. THF);

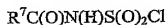
- (v) for compounds of formula I in which R^4 represents
 $-N(H)S(O)_2N(H)C(O)R^7$ and R^7 is as hereinbefore defined, reaction of a
 compound of formula IX,

20



IX

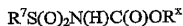
wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as hereinbefore defined with a compound of formula X,



X

wherein R^7 is as hereinbefore defined, for example at or around room temperature in the presence of a suitable base (e.g. sodium hydroxide or triethylamine) and a suitable organic solvent (e.g. benzene or dichloromethane);

(vi) for compounds of formula I in which R^4 represents $-N(H)C(O)N(H)S(O)_2R^7$ and R^7 is as hereinbefore defined, reaction of a compound of formula IX as hereinbefore defined with a compound of formula XI,



XI

wherein R^x represents C_{1-2} alkyl and R^7 is as hereinbefore defined, for example at or around room temperature in the presence of a suitable organic solvent (e.g. dichloromethane);

14

(vii) for compounds of formula I in which R^4 represents $-N(H)C(O)N(H)S(O)_2R^7$ and R^7 is as hereinbefore defined, reaction of a compound of formula IX as hereinbefore defined with an isocyanate compound of formula XII,

5



wherein R^7 is as hereinbefore defined, for example at or around room temperature in the presence of a suitable organic solvent (e.g. dichloromethane);

10

(viii) for compounds of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ and R^6 represents C_{1-6} alkylamino, reaction of a compound of formula II as hereinbefore defined with an isocyanate compound of formula XIII,

15



wherein R^{6b} is C_{1-6} alkyl, for example at or around room temperature in the presence of a suitable base (e.g. sodium hydroxide or potassium hydroxide and an appropriate organic solvent (e.g. acetone or acetonitrile); or

20

(ix) for compounds of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ and R^6 represents di- C_{1-6} alkylamino, reaction of a corresponding compound of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ and R^6 represents C_{1-6} alkoxy with an amine of formula XIV,

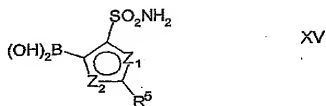
25



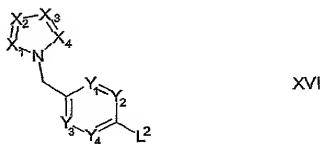
30

wherein R^{60} and R^{6d} independently represent C_{1-6} alkyl, for example at above room temperature (e.g. at between 70°C and 100°C) in the presence of an appropriate organic solvent (e.g. toluene).

- 5 Compounds of formula II may be prepared by reaction of a compound of formula XV,



- 10 wherein R^5 , Z^1 and Z^2 are as hereinbefore defined, or a N-protected derivative thereof, with a compound of formula XVI,



- 15 wherein L^2 represents a suitable leaving group, such as trimethylsulphonate, or halo, such as iodo or bromo, and X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 and Y_4 are as hereinbefore defined, for example in the presence of an appropriate coupling catalyst system (e.g. a palladium catalyst, such as $\text{Pd}(\text{PPh}_3)_4$ or $\text{Pd}(\text{OAc})_2/\text{ligand}$ (wherein the ligand may be, for example, PPh_3 , $\text{P}(\text{o-Tol})_3$ or 1,1'-bis(diphenylphosphino)ferrocene)) and a suitable base (e.g. sodium hydroxide, sodium carbonate, potassium carbonate, cesium carbonate, triethylamine or di-*iso*-propylamine), as well as a suitable solvent system (e.g. toluene, ethanol, dimethoxymethane, dimethylformamide, ethylene
- 20

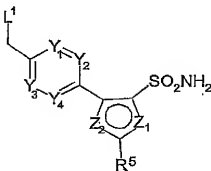
glycol dimethyl ether, water, dioxane or mixtures thereof). This reaction may be carried out at above room temperature (e.g. at the reflux temperature of the solvent system that is employed). If a protected version of a compound of formula XV is employed, this reaction may be followed by deprotection of the SO_2NH -group under standard conditions, for example as described hereinafter.

Compounds of formula II may alternatively be prepared by reaction of a compound of formula XVII,



XVII

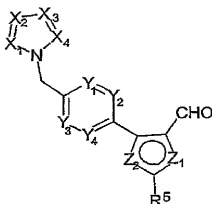
wherein X_1 , X_2 , X_3 and X_4 are as hereinbefore defined with a compound of formula XVIII,



XVIII

wherein Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 , R^5 and L^1 are as hereinbefore defined (L^1 , in particular, may represent bromo), or a N-protected derivative thereof, for example at around or below room temperature in the presence of a suitable base (e.g. potassium hydroxide) and an appropriate organic solvent (e.g. DMSO). If a protected version of a compound of formula XVIII is

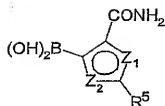
- employed, this reaction may be followed by deprotection of the SO_2NH -group under standard conditions, for example as described hereinafter. Additionally, compounds of formula II in which Z_1 is $-\text{CH}=\text{CH}-$ and Z_2 is $-\text{CH}-$ may be prepared in this way, for example according, or analogously, to processes described in *inter alia* US patent number 5,312,820. Further, compounds of formula II in which Z_1 is $-\text{S}-$ and Z_2 is $-\text{CH}-$ may be prepared in this way for example according, or analogously, to processes described in *inter alia* UK patent application GB 2281298.
- 10 Compounds of formula V may be prepared by oxidation of a compound of formula XIX,



XIX

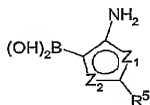
- 15 wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as hereinbefore defined, for example under standard oxidation conditions in the presence of a suitable oxidising agent, such as potassium permanganate or chromium (VI) oxide.
- 20 Compounds of formulae VII and IX may be prepared by reaction of a compound of formula XVI as hereinbefore defined with (in the case of a compound of formula VII) a compound of formula XX,

18



XX

or (in the case of a compound of formula IX) a compound of formula XXI,

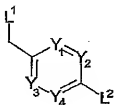


XXI

5

wherein, in both cases, Z_1 , Z_2 and R^5 are as hereinbefore defined, or N-protected derivatives thereof, for example under similar conditions to those described hereinbefore for preparation of compounds of formula II (first process). If protected versions of compounds of formulae XX and XXI are employed, these reactions may be followed by deprotection of the NH-group under standard conditions (e.g. acid hydrolysis).

Compounds of formula XVI may be prepared by standard techniques, for example by way of reaction of a compound of formula XVII as hereinbefore defined with a compound of formula XXII,

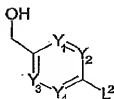


XXII

wherein Y_1 , Y_2 , Y_3 , Y_4 , L^1 and L^2 are as hereinbefore defined, for example under similar conditions to those described hereinbefore in respect of preparation of compounds of formula II (second process).

- 5 Compounds of formula XVIII are known in the art. For example, they may be prepared according, or analogously, to processes described in *inter alia* US patent number 5,312,820, UK patent application GB 2281298, and/or by reaction of a compound of formula XV as hereinbefore defined with a compound of formula XXIII,

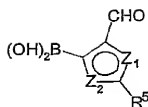
10



XXIII

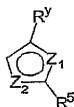
- wherein Y_1 , Y_2 , Y_3 , Y_4 and L^2 are as hereinbefore defined, for example under similar conditions to those described hereinbefore in respect of
- 15 preparation of compounds of formula II (first process), followed by conversion of the OH group in the resultant intermediate to an appropriate leaving group, L^1 (e.g., in the case where L^1 is bromo, conversion may be carried out by reaction with CBr_4 , for example at or around room temperature in the presence of a base (e.g. triphenylphosphine) and a
- 20 suitable organic solvent (e.g. DMF)).

Compounds of formula XIX may be prepared by reaction of a compound of formula XVI as hereinbefore defined with a compound of formula XXIV,



XXIV

- wherein Z_1 , Z_2 and R^5 are as hereinbefore defined, or a protected (at the aldehyde part) derivative thereof, for example under similar conditions to those described hereinbefore for preparation of compounds of formula II (first process). If a protected version of a compound of formula XXIV is employed, this reaction may be followed by deprotection of the CHO-group under standard conditions (e.g. acid hydrolysis).
- 10 Compounds of formulae XV, XX, XXI and XXIV and protected derivatives thereof may be prepared by reaction of a corresponding compound of formula XXV,



XXV

- 15 wherein R^Y represents $-S(O)_2NH_2$, $-C(O)NH_2$, $-NH_2$ or $-CHO$ (as appropriate) and R^5 , Z_1 and Z_2 are as hereinbefore defined, or an appropriate protected derivative thereof, with a reagent system that will enable the introduction of the $-B(OH)_2$ into the appropriate ring system. Suitable reagent systems include trialkylborates (e.g. tri-*iso*-propylborate). Such reactions may be carried out, for example, at low temperature (e.g. between $-100^\circ C$ and $0^\circ C$, e.g. between $-80^\circ C$ (such as $-78^\circ C$) and $-10^\circ C$ (such as $-20^\circ C$)) in the presence of a suitable base (e.g. *n*-butyl lithium) and an
- 20

appropriate organic solvent (e.g. THF), followed by acid hydrolysis (e.g. in the presence of dilute HCl).

Compounds of formula XXV are available using known techniques. For example:

- (a) Compounds of formula XXV in which R^y represents $-S(O)_2NH_2$, $-C(O)NH_2$ or $-CHO$, and protected derivatives thereof, may be prepared by reaction of a compound of formula XXVI,



XXVI

wherein R^y represents $-S(O)_2NH_2$, $-C(O)NH_2$ or $-CHO$ and Z_1 and Z_2 are as hereinbefore defined, or a protected derivative thereof, with a compound of formula XXVII,



XXVII

wherein L^3 represents a suitable leaving group (such as toluenesulphonate, benzenesulphonate, methanesulphonate or halo, such as bromo or iodo) and R^5 is as hereinbefore defined, for example at below room temperature (e.g. between around -35°C and around -85°C), in the presence of a suitable base (e.g. *n*-butyl lithium) and an appropriate solvent (e.g. THF).

- (b) Compounds of formula XXV in which R^y is $-S(O)_2NH_2$ and N-protected derivatives thereof, may be prepared by reaction of an appropriate compound of formula XXVIII,



XXVIII

5

wherein R^5 , Z_1 and Z_2 are as hereinbefore defined with an appropriate reagent for introduction of a $-S(O)_2NH_2$ group into the appropriate ring system (for example chlorosulphonic acid, or thionyl chloride in the presence of a suitable strong base (e.g. butyl lithium)), followed by reaction of the resultant intermediate with ammonia, or a protected derivative thereof (e.g. *tert*-butylamine), under conditions that are well known to those skilled in the art.

10

- 15 (c) Certain protected derivatives (e.g. alkyl, such as C_{1-6} alkyl, for example *tert*-butyl, protected derivatives) of compounds of formula XXV in which R^y represents $-C(O)NH_2$ may be prepared by reaction of a compound of formula XXVIII as hereinbefore defined, with a compound of formula XXIX,

20

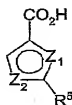


XXIX

wherein R^Z represents an appropriate protecting group, such as an alkyl group, including C_{1-6} alkyl, e.g. *tert*-butyl, for example at low temperature (e.g. $-78^\circ C$ to around $0^\circ C$), in the presence of a suitable base (e.g. *n*-butyl lithium) and an appropriate solvent (e.g. THF).

25

- (d) Certain protected derivatives (e.g. alkyl, such as C_{1-6} alkyl, for example *tert*-butyl, protected derivatives) of compounds of formula XXV in which R^y represents $-C(O)NH_2$ may also be prepared by reaction of a compound of formula XXX,



XXX

wherein R^5 , Z_1 and Z_2 are as hereinbefore defined with a protected (e.g. an (e.g. C_{1-6}) alkyl, such as *tert*-butyl-protected) derivative of ammonia (e.g. *tert*-butylamine) under standard coupling conditions (see, for example, those described hereinbefore for preparation of compounds of formula I (process step (iii))). Compounds of formula XXX are known in the art or may be prepared by way of standard techniques, for example oxidation of a corresponding compound of formula XXV in which R^y is $-CHO$ e.g. under those conditions described hereinbefore for preparation of compounds of formula V.

- (e) Compounds of formula XXV in which R^y is $-CHO$, Z_1 represents $-CH=CH-$ and Z_2 represents $-CH-$, and protected derivatives thereof, may be prepared by reaction of a compound of formula XXVIII in which Z_1 represents $-CH=CH-$ and Z_2 represents $-CH-$ with an appropriate reagent system for the introduction of an aldehyde group into the benzene ring (e.g. $TiCl_4/CHCl_3$, $SnCl_4/CH_2Cl_2$ or 1,3,5,7-azaadamantane/TFA) under standard reaction conditions, followed by (if appropriate) protection of the resultant benzaldehyde under standard conditions.

(f) Compounds of formula XXV in which R^y is $-NH_2$, Z_1 represents $-CH=CH-$ and Z_2 represents $-CH-$, and N-protected derivatives thereof, may be prepared by nitration of a compound of formula XXVIII in which Z_1 represents $-CH=CH-$ and Z_2 represents $-CH-$, followed by reduction of the resultant nitrobenzene and (if appropriate) protection of the resultant aminobenzene, all of which steps may be carried out under standard conditions.

Compounds of formulae III, IV, VI, VIII, X, XI, XII, XIII, XIV, XVII, XXII, XXIII, XXVI, XXVII, XXVIII and XXIX are either commercially available, are known in the literature, or may be obtained either by analogy with the processes described herein, or by conventional synthetic procedures, in accordance with standard techniques, from readily available starting materials using appropriate reagents and reaction conditions.

Compounds of the invention may be isolated from their reaction mixtures using conventional techniques.

It will be appreciated by those skilled in the art that, in the processes described above and hereinafter, the functional groups of intermediate compounds may need to be protected by protecting groups.

Functional groups that it is desirable to protect include sulphonamido, amido, amino and aldehyde. Suitable protecting groups for sulphonamido, amido and amino include *tert*-butoxycarbonyl, benzyloxycarbonyl, 2-trimethylsilylethoxycarbonyl (Teoc) or *tert*-butyl. Suitable protecting groups for aldehyde include alcohols, such as methanol or ethanol, and diols, such as 1,3-propanediol or, preferably, 1,2-ethanediol (so forming a cyclic acetal).

The protection and deprotection of functional groups may take place before or after a reaction in the above-mentioned schemes.

Protecting groups may be removed in accordance with techniques that are well known to those skilled in the art and as described hereinafter. For example, protected compounds/intermediates described herein may be converted chemically to unprotected compounds using standard deprotection techniques (e.g. using trifluoroacetic acid, sulfuric acid, toluenesulfonic acid or boron trichloride).

Persons skilled in the art will appreciate that, in order to obtain compounds of the invention in an alternative, and, on some occasions, more convenient, manner, the individual process steps mentioned hereinbefore may be performed in a different order, and/or the individual reactions may be performed at a different stage in the overall route (i.e. substituents may be added to and/or chemical transformations performed upon, different intermediates to those mentioned hereinbefore in conjunction with a particular reaction). This may negate, or render necessary, the need for protecting groups.

The type of chemistry involved will dictate the need, and type, of protecting groups as well as the sequence for accomplishing the synthesis.

The use of protecting groups is fully described in "Protective Groups in Organic Chemistry", edited by J W F McOmie, Plenum Press (1973), and "Protective Groups in Organic Synthesis", 3rd edition, T.W. Greene & P.G.M. Wutz, Wiley-Interscience (1999).

Medical and Pharmaceutical Uses

Compounds of the invention are useful because they possess pharmacological activity. The compounds of the invention are therefore
5 indicated as pharmaceuticals.

According to a further aspect of the invention there is thus provided the compounds of the invention for use as pharmaceuticals.

10 In particular, compounds of the invention are agonists of AngII, more particularly, are agonists of the AT₂ receptor, and, especially, are selective agonists of that sub-receptor, for example as may be demonstrated in the tests described below.

15 The compounds of the invention are thus expected to be useful in those conditions in which endogenous production of AngII is deficient and/or where an increase in the effect of AngII is desired or required.

The compounds of the invention are further expected to be useful in those
20 conditions where AT₂ receptors are expressed and their stimulation is desired or required.

The compounds of the invention are further indicated in the treatment of conditions characterised by vasoconstriction, increased cell growth and/or
25 differentiation, increased cardiac contractility, increased cardiovascular hypertrophy, and/or increased fluid and electrolyte retention.

The compounds of the invention are further indicated in the treatment of stress-related disorders, and/or in the improvement of microcirculation
30 and/or mucosa-protective mechanisms.

Thus, compounds of the invention are expected to be useful in the treatment of disorders, which may be characterised as indicated above, and which are of, for example, the gastrointestinal tract, the cardiovascular system, the respiratory tract, the kidneys, the eyes, the female reproductive (ovulation) system and the central nervous system (CNS).

Disorders of the gastrointestinal tract that may be mentioned include oesophagitis, Barrett's oesophagus, gastric ulcers, duodenal ulcers, dyspepsia (including non-ulcer dyspepsia), gastro-oesophageal reflux, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), pancreatitis, hepatic disorders (such as hepatitis), gall bladder disease, multiple organ failure (MOF) and sepsis. Other gastrointestinal disorders that may be mentioned include xerostomia, gastritis, gastroparesis, hyperacidity, disorders of the biliary tract, coeliac, Crohn's disease, ulcerative colitis, diarrhoea, constipation, colic, dysphagia, vomiting, nausea, indigestion and Sjögren's syndrome.

Disorders of the respiratory tract that may be mentioned include inflammatory disorders, such as asthma, obstructive lung diseases (such as chronic obstructive lung disease), pneumonitis, pulmonary hypertension and adult respiratory distress syndrome.

Disorders of the kidneys that may be mentioned include renal failure, nephritis and renal hypertension.

Disorders of the eyes that may be mentioned include diabetic retinopathy, premature retinopathy and retinal microvascularisation.

Disorders of the female reproductive system that may be mentioned include ovulatory dysfunction.

Cardiovascular disorders that may be mentioned include hypertension,
5 cardiac hypertrophy, cardiac failure, atherosclerosis, arterial thrombosis,
venous thrombosis, endothelial dysfunction, endothelial lesions, post-
balloon dilatation stenosis, angiogenesis, diabetic complications,
microvascular dysfunction, angina, cardiac arrhythmias, claudication
intermittens, preeclampsia, myocardial infarction, reinfarction, ischaemic
10 lesions, erectile dysfunction and neointima proliferation.

Disorders of the CNS that may be mentioned include cognitive
dysfunctions, dysfunctions of food intake (hunger/satiety) and thirst, stroke,
cerebral bleeding, cerebral embolus and cerebral infarction.

15 Compounds of the invention may also be useful in the modulation of growth
metabolism and proliferation, for example in the treatment of hypertrophic
disorders, prostate hyperplasia, autoimmune disorders, psoriasis, obesity,
neuronal regeneration, the healing of ulcers, inhibition of adipose tissue
20 hyperplasia, stem cell differentiation and proliferation, cancer (e.g. in the
gastrointestinal tract, lung cancer, etc), apoptosis, tumours (generally) and
hypertrophy, diabetes, neuronal lesions and organ rejection.

The compounds of the invention are indicated both in the therapeutic and/or
25 prophylactic treatment of the above conditions.

According to a further aspect of the present invention, there is provided a
method of treatment of a condition in which endogenous production of
AngII is deficient, and/or a condition where an increase in the effect of
30 AngII is desired or required, and/or a condition where AT2 receptors are

expressed and their stimulation is desired or required, which method comprises administration of a therapeutically effective amount of a compound of the invention to a person suffering from, or susceptible to, such a condition.

5

The compounds of the invention will normally be administered orally, intravenously, subcutaneously, buccally, rectally, dermally, nasally, tracheally, bronchially, by any other parenteral route or *via* inhalation, in a pharmaceutically acceptable dosage form.

10

When the condition to be treated is multiple organ failure, preferred routes of administration are parenteral (e.g. by injection). Otherwise, the preferred route of administration for compounds of the invention is oral.

15 The compounds of the invention may be administered alone, but are preferably administered by way of known pharmaceutical formulations, including tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions or suspensions for parenteral or intramuscular administration, and the like.

20

Such formulations may be prepared in accordance with standard and/or accepted pharmaceutical practice.

25 According to a further aspect of the invention there is thus provided a pharmaceutical formulation including a compound of the invention, in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier.

Compounds of the invention may also be administered in combination with other AT2 agonists that are known in the art, as well as in combination with

AT1 receptor antagonists that are known in the art, such as losartan, or in combination with an inhibitor of angiotensin converting enzyme (ACE).

According to a further aspect of the invention, there is provided a combination product comprising:

- (A) a compound of the invention; and
 - (B) an AT1 receptor antagonist, or an ACE inhibitor,
- wherein each of components (A) and (B) is formulated in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier.

Such combination products provide for the administration of compound of the invention in conjunction with an AT1 receptor antagonist, or an ACE inhibitor, and may thus be presented either as separate formulations, wherein at least one of those formulations comprises compound of the invention, and at least one comprises AT1 receptor antagonist, or ACE inhibitor, or may be presented (i.e. formulated) as a combined preparation (i.e. presented as a single formulation including compound of the invention and AT1 receptor antagonist or ACE inhibitor).

Thus, there is further provided:

- (1) a pharmaceutical formulation including a compound of the invention and an AT1 receptor antagonist, or an ACE inhibitor, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier; and

- (2) a kit of parts comprising components:
 - (a) a pharmaceutical formulation including a compound of the invention, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier; and

- (b) a pharmaceutical formulation including an AT1 receptor antagonist, or an ACE inhibitor, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier, which components (a) and (b) are each provided in a form that is suitable for administration in conjunction with the other.

Depending upon the disorder and patient to be treated and the route of administration, the compounds of the invention may be administered at varying doses.

Although doses will vary from patient to patient, suitable daily doses are in the range of about 1 to 1000 mg per patient, administered in single or multiple doses. More preferred daily doses are in the range 2.5 to 250 mg per patient.

Individual doses of compounds of the invention may be in the range 1 to 100 mg.

In any event, the physician, or the skilled person, will be able to determine the actual dosage which will be most suitable for an individual patient, which is likely to vary with the condition that is to be treated, as well as the age, weight, sex and response of the particular patient to be treated. The above-mentioned dosages are exemplary of the average case; there can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Compounds of the invention have the advantage that they bind selectively to, and exhibit agonist activity at, the AT2 receptor. By compounds which "bind selectively" to the AT2 receptor, we include that the affinity ratio for

the relevant compound (AT₂:AT₁) is at least 5:1, preferably at least 10:1 and more preferably at least 20:1.

The compounds of the invention may also have the advantage that they may be more efficacious than, be less toxic than, be longer acting than, be more potent than, produce fewer side effects than, be more easily absorbed than, and/or have a better pharmacokinetic profile (e.g. higher oral bioavailability and/or lower clearance) than, and/or have other useful pharmacological, physical, or chemical properties over, compounds known in the prior art.

Biological Tests

The following test procedures may be employed.

Test A

Receptor Binding Assay using Rat Liver Membrane AT₁ Receptor

Rat liver membranes were prepared according to the method of Dudley *et al* (*Mol. Pharmacol.* (1990) 38, 370). Binding of [¹²⁵I]Ang II to membranes was conducted in a final volume of 0.5 mL containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA (bovine serum albumin), liver homogenate corresponding to 5 mg of the original tissue weight, [¹²⁵I]Ang II (70 000 cpm, 0.03 nM) and variable concentrations of test substance. Samples were incubated at 25°C for 1 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets using a Brandel cell harvester. The filters were washed with 4 × 2 mL of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a gamma counter. The characteristics of the Ang II binding AT₁ receptor were determined by using six different concentrations (0.03-5 nmol/L) of the labeled [¹²⁵I]AngII. Non-specific binding was determined in the presence of 1 μM Ang II. The specific binding was determined by subtracting the non-specific binding from the total bound [¹²⁵I]AngII. The

dissociation constant ($K_d = 1.7 \pm 0.1$ nM, $[L] = 0.057$ nM) was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK). The binding data were best fitted with a one-site fit. All experiments were performed at least in triplicate.

Test B

Receptor Binding Assay using Porcine Myometrial Membrane AT₂ Receptor

Myometrial membranes were prepared from porcine uteri according to the method by Nielsen *et al* (*Clin. Exp. Pharm. Phys.* (1997) 24, 309). Any possible interference that may be exhibited by binding of compound to AT₁ receptors was blocked by addition of 1 μ M of a selective AT₁ inhibitor. Binding of [¹²⁵I]Ang II to membranes was conducted in a final volume of 0.5 mL containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA, homogenate corresponding to 10 mg of the original tissue weight, [¹²⁵I]Ang II (70 000 cpm, 0.03 nM) and variable concentrations of test substance. Samples were incubated at 25°C for 1 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets using a Brandel cell harvester. The filters were washed with 3 \times 3 mL of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured using a gamma counter. The characteristics of the Ang II binding AT₂ receptor was determined by using six different concentrations (0.03-5 nmol/L) of the labeled [¹²⁵I]Ang II. Non-specific binding was determined in the presence of 1 μ M Ang II. The specific binding was determined by subtracting the non-specific binding from the total bound [¹²⁵I]Ang II. The dissociation constant ($K_d = 0.7 \pm 0.1$ nM, $[L] = 0.057$ nM) was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK). The binding data were best fitted with a one-site fit. All experiments were performed at least in triplicate.

Test C

Duodenal Mucosal Alkaline Secretion Assay

Compounds were exposed to the duodenal mucosa in barbiturate-
5 anaesthetised rats prepared for *in situ* titration of duodenal mucosal alkaline
secretion, according to the methodology described by Flemström *et al* in
Am. J. Physiol. (1982) **243**, G348.

The invention is illustrated by way of the following examples.

10

Example 1

N-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthio- phene-2-sulfonamide

15 (a) N-tert-Butylthiophene-2-sulfonamide

Thiophene-2-sulfonyl chloride (15 g, 0.082 mol) was dissolved in CHCl₃
(200 mL) under N₂ atmosphere and then cooled to 0°C. *tert*-Butylamine
(25.9 mL, 0.246 mol) dissolved in CHCl₃ (50 mL) was then added dropwise
to the reaction mixture. The reaction mixture was stirred for 1 h at room
20 temperature and then at reflux for 10 min. Toluene (700 mL) was added
and the organic phase was washed with water (3 x 50 mL), dried, and
concentrated *in vacuo*. The sub-title product was used without further
purification in the next step.

¹H NMR δ(CDCl₃): 7.60(1H, dd, J=1.3, 3.8 Hz), 7.53(1H, dd, J=1.3, 5.0
25 Hz), 7.02(1H, dd, J=5.0, 3.8 Hz), 5.13(1H, m), 1.24 (9H, m)

¹³C NMR δ(CDCl₃): 145.0, 131.7, 131.2, 127.0, 55.1, 29.9

(b) 5-iso-Butyl-N-tert-butylthiophene-2-sulfonamide

N-tert-Butylthiophene-2-sulfonamide (10 g, 0.046 mol, see step (a) above)
30 was dissolved in THF (85 mL) under N₂ and then cooled to -78°C. *n*-BuLi

(1.6 M, 76.9 mL, 0.12 mol) was added *via* a syringe. The reaction mixture was stirred at -78°C for 30 min. and then at -40°C for 2 h. Iodo-2-methylpropane (10.5 mL, 0.09 mol) was added dropwise to the reaction mixture. The reaction mixture was stirred overnight at room temperature.

- 5 The reaction was quenched with NH_4Cl (aq.) and extracted with EtOAc. The combined organic phase was washed with brine and dried and concentrated *in vacuo*. The crude product was purified on column chromatography (hexanes:EtOAc (10:1)) to give the sub-title compound in 55% yield (7.0 g, 0.025 mol).

- 10 ^1H NMR $\delta(\text{CDCl}_3)$: 7.43(1H, d, $J=3.6$ Hz), 6.67(1H, d, $J=3.8$ Hz), 4.83(1H, m), 2.67(2H, d, $J=7$ Hz), 1.88 (1H, m), 1.26(9H, m), 0.93(6H, $J=6.6$ Hz).
 ^{13}C NMR $\delta(\text{CDCl}_3)$: 145.0, 131.7, 131.2, 127.0, 55.1, 29.9

(c) 5-iso-Butyl-2-(N-tert-butylaminosulfonyl)thiophene-3-boronic acid

- 15 5-iso-Butyl-N-tert-butylthiophene-2-sulfonamide (10.6 g, 0.039 mol, see step (b) above) was dissolved in THF (165 mL) under N_2 and then cooled to -78°C . *n*-BuLi (1.6 M, 60.19 mL, 0.096 mol) was added *via* a syringe. The reaction mixture was stirred at -20°C for 4 h. The tri-*iso*-propylborate (13.3 mL, 0.058 mol) was then added *via* a syringe and the reaction mixture was
20 stirred overnight at room temperature. The reaction was quenched with 2 M HCl (20 mL). The organic phase was separated and the water phase was extracted with EtOAc (3 x 100 mL). The combined organic phase was washed with brine, dried and concentrated *in vacuo*. The product was used without further purification.
25 MS(ESI $^+$) m/z : 236.8

(d) 1-(4-Bromobenzyl)-1H-imidazole

- Dimethyl sulphoxide (20 mL; dried over 4A molecular sieve) was added to potassium hydroxide (2.24 g, 0.04 mol, crushed pellets) and the mixture
30 was stirred for 5 min. Imidazole (0.5718 g, 0.0084 mol) was then added

and the mixture was stirred for 2 h. 4-Bromobenzyl bromide (3.25 g, 0.013 mol) was added and the mixture was cooled briefly and stirred for a further 1 h before water (20 mL) was added. The mixture was extracted with ether (3 × 100 mL) and each extract was washed with water (3 × 50 mL). The combined ether layers were dried over CaCl₂ and the solvent was removed *in vacuo*. The residue was chromatographed on silica gel with CHCl₃:MeOH (30:1) plus 0.05% formic acid as eluent to give the sub-title product (1.275 g, yield: 53%).

¹H NMR δ(CDCl₃): 7.73(3H, m), 7.28(3H, m), 7.15(1H, m), 5.30(2H, s)

¹³C NMR δ(CDCl₃): 136.8, 134.8, 131.5, 129.3, 128.4, 121.5, 118.7, 49.4.

MS(ESI⁺) m/z: 236.8

(e) 3-(4-Imidazol-1-ylmethylphenyl)-5-iso-butyl-N-tert-butylthiophene-2-sulfonamide

5-iso-Butyl-2-(N-tert-butylaminosulfonyl)thiophene-3-boronic acid (200.5 mg, 0.628 mmol, see step (c) above), 1-(4-bromobenzyl)-1H-imidazole (98.8 mg, 0.416 mmol, see step (d) above), toluene (15 mL), ethanol (15 mL), NaOH (1.0M, 1.5 mL, 1.5 mmol) and Pd(PPh₃)₄ (14.5 mg, 0.125 mmol) were mixed under N₂. The mixture was warmed to reflux for 2 h.

The mixture was diluted with EtOAc (50 mL), washed with water and brine, and dried over MgSO₄. The solvent was removed and the residue was separated by column chromatography with chloroform:methanol (20:1) as an eluent to give 113.9 mg of the sub-title compound (yield: 63.27%).

IR(neat): 3060, 2996, 1507 cm⁻¹

¹H NMR δ(CDCl₃): 7.39(1H, s), 7.35(2H, d, J=8.1 Hz), 6.98(2H, d, J=8.1 Hz), 6.96(1H, s), 6.84(H, s), 6.47(H, s), 4.91(2H, s), 3.96(1H, s), 2.72(H, brs), 2.42(2H, d, J=7.1 Hz), 1.64(1H, m), 0.73(9H, s), 0.72(6H, d, J=6.9 Hz)

¹³C NMR δ(CDCl₃): 148.6, 142.3, 137.2, 136.2, 135.1, 129.7, 129.4, 128.8, 127.4, 119.2, 54.6, 50.6, 39.2, 30.5, 29.5, 22.1

MS(ESI⁺) *m/z*: 431.9 Anal. Calcd. for C₂₂H₂₉N₃O₂S₂: C, 58.8; H, 7.0; N, 9.4. Found: C, 58.7.0; H, 6.7; N, 9.1.

(f) 3-(4-Imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

- 5 Trifluoroacetic acid was added (2 mL) to 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butyl-*N-tert*-butylthiophene-2-sulfonamide (113 mg, 0.2618 mmol, see step (e) above) and one drop (*ca.* 0.05 mL) of anisole (*ca.* 0.05 mL) was added to the mixture. The reaction mixture was stirred under N₂ atmosphere for 30 h and then evaporated and co-evaporated with
10 acetonitrile until TLC showed that it was pure. The crude product was used directly in the next step without further purification.

¹H NMR δ(CDCl₃): 7.70(1H, s), 7.57(2H, d, J=8.1 Hz), 7.19(2H, d, J=8.1 Hz), 7.10(1H, s), 6.93(H, s), 6.73(H, s), 5.14(2H, s), 2.67(2H, d, J=7.1 Hz), 2.62(H, brs), 1.94(1H, m), 0.97(6H, d, J=6.6 Hz)

- 15 ¹³C NMR δ(CDCl₃): 148.4, 142.9, 137.2, 136.2, 134.6, 129.7, 129.3, 128.8, 127.3, 119.2, 50.6, 39.2, 30.5 22.1

MS(ESI⁺) *m/z*: 375.9

- 20 (g) *N*-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

- The crude 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide from step (f) above was dissolved in pyridine (2 mL, dried over 4Å molecular sieve). Pyrrolidinopyridine (40.52 mg, 0.2618 mmol) and butyl chloroformate (363.5 mg, 0.339 mL) were added to the mixture.
25 The mixture was stirred overnight under a N₂ atmosphere at room temperature. Evaporation and co-evaporation with acetonitrile to remove the solvents and purification on column chromatography with 10% MeOH in chloroform as eluent gave the title compound (57.8 mg, 0.1217 mmol) in a 46.5% yield (over the last two steps).
30 IR(neat): 3555.8, 3120.3, 2955.9, 1694.2, 1268.5 cm⁻¹

^1H NMR $\delta(\text{CDCl}_3)$: 7.96(1H, s), 7.57(2H, d, $J=7.9$ Hz), 7.10(2H, d, $J=7.9$ Hz), 6.89(H, s), 6.85(H, s), 6.74(H, s), 5.16(2H, s), 4.03(2H, t, $J=6.6$ Hz), 2.71(2H, d, $J=7.1$ Hz), 1.94(1H, m), 1.51(2H, m), 1.25(2H, m), 0.98(6H, d, $J=6.6$ Hz), 0.87(3H, t, $J=7.4$ Hz)

5 ^{13}C NMR $\delta(\text{CDCl}_3)$: 152.5, 158.4, 143.9, 136.4, 134.6, 133.0, 129.8, 128.9, 127.3, 125.6, 119.6, 65.9, 51.2, 39.3, 30.6, 30.4, 22.3, 18.9, 13.7

MS(ESI $^+$) m/z : 476.0 Anal. Calcd for $\text{C}_{23}\text{H}_{29}\text{N}_3\text{O}_4\text{S}_2 \cdot \text{H}_2\text{O}$: C, 56.0; H, 6.3; N, 8.5. Found: C, 56.4; H, 6.2; N, 8.6

10 Example 2

N-iso-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

30 mg of crude 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide (see Example 1(f) above) was dissolved in pyridine (1 mL, dried over 4Å molecular sieve) and cooled on ice. Pyrrolidinopyridine (11.8 mg, 0.080 mmol) and *iso*-butyl chloroformate (103.6 μL , 0.80 mmol) were then added to the mixture. The mixture was stirred overnight under a N_2 atmosphere at 50°C. Evaporation and co-evaporation with acetonitrile to remove the solvents, followed by purification by column chromatography using 10% MeOH in CHCl_3 as eluent gave the title compound (27 mg, 0.057 mmol) in 71% yield.

15 ^1H NMR $\delta(\text{CD}_3\text{OD})$: 8.18 (brs, 1H), 7.58 (d, $J=7.9$ Hz, 2H), 7.32 (d, $J=7.9$ Hz, 2H), 7.29 (brs, 1H), 7.17 (brs, 1H), 6.82 (s, 1H), 5.30 (s, 2H), 3.70 (d, $J=6.6$ Hz, 2H), 2.72 (d, $J=7.1$ Hz, 2H), 1.93 (m, 1H), 1.76 (m, 1H), 0.99 (d, $J=6.6$ Hz, 6H), 0.81 (d, $J=7.4$ Hz, 6H)

25 ^{13}C NMR $\delta(\text{CD}_3\text{OD})$: 155.7, 150.5, 145.2, 137.0, 136.4, 135.6, 131.0, 130.5, 128.9, 126.6, 121.8, 73.0, 52.2, 40.0, 31.9, 29.1, 22.6, 19.3

MS (ESI $^+$) m/z : 476.0

Example 3N-iso-Propyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

100 mg of crude 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide (see Example 1(f) above) was dissolved in pyridine (4 mL, dried over 4Å molecular sieve) and cooled on ice. Pyrrolidinopyridine (39.5 mg, 0.266 mmol) and iso-propyl chloroformate (1M in toluene, 2.66 mL, 2.66 mmol) were then added to the mixture. The mixture was stirred overnight under N₂ atmosphere at 50°C. Evaporation and co-evaporation with acetonitrile to remove the solvents, followed by purification using preparative LC/MS (30% acetonitrile to pure acetonitrile, reverse phase) gave the title compound (52.6 mg, 0.114 mmol).

¹H NMR δ(CD₃OD): 8.16 (brs, 1H), 7.58 (d, J = 8.1 Hz, 2H), 7.34 (d, J = 8.1 Hz, 2H), 7.32 (s, 1H), 7.18 (s, 1H), 6.84 (s, 1H), 5.32 (s, 2H), 4.72 (sep, J = 6.3 Hz, 1H), 2.73 (d, J = 7.1 Hz, 2H), 1.94 (m, 1H), 1.09 (d, J = 6.3 Hz, 6H), 1.00 (d, J = 6.6 Hz, 6H)

¹³C NMR δ(CD₃OD): 155.5, 151.0, 145.6, 137.2, 136.2, 135.0, 131.0, 130.5, 128.9, 126.8, 122.0, 70.5, 52.1, 40.0, 31.9, 22.6, 22.1

MS (ESI⁺) m/z: 462.0

Example 4N-(Butoxyacetyl)-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

CDI (1,1'-carbonyl-diimidazole, 129.5 mg, 0.80 mmol) was added to a solution of butoxyacetic acid (103.8 μL, 0.80 mmol) in dry THF (4 mL). The mixture was stirred at 50°C for 2.5 h. A solution of 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide (see Example 1(f) above; 100 mg) and DBU (1,8-diazabicyclo[5.4.0]undec-7-ene, 19.9 μL, 0.133 mmol) in dry THF (4 mL) was added to the reaction mixture. The reaction mixture was then stirred overnight at 50°C. MeOH (20 mL) was

added to the reaction mixture, which was then concentrated *in vacuo*. The residue was dissolved in EtOAc (100 mL) and then washed with water (3 x 50 mL). The organic phase was dried and evaporated. The crude product was purified by column chromatography using 10% MeOH in CHCl₃ as eluent to give the title compound (27 mg, 0.057 mmol).

¹H NMR δ(CD₃OD): 7.96 (brs, 1H), 7.66 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 8.3 Hz, 2H), 7.21 (brs, 1H), 7.07 (brs, 1H), 6.79 (s, 1H), 5.26 (s, 2H), 3.67 (s, 2H), 3.40 (t, *J* = 6.8 Hz, 2H), 2.66 (d, *J* = 6.9 Hz, 2H), 1.88 (m, 1H), 1.48 (m, 2H), 1.29 (m, 4H), 0.96 (d, *J* = 6.6 Hz, 6H), 0.82 (t, *J* = 7.3 Hz, 3H)

¹³C NMR δ(CD₃OD): 178.3, 148.3, 143.4, 138.0, 137.3, 136.6, 131.1, 130.3, 128.5, 128.2, 127.6, 121.4, 72.4, 72.3, 51.7, 39.9, 32.2, 31.8, 30.7, 22.6, 20.1, 14.3

MS (ESI⁺) *m/z*: 490.1

Example 5

N-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-butylthiophene-2-sulfonamide

(a) 5-Butylthiophene-2-sulfonic acid *tert*-butylamide

N-tert-Butylthiophene-2-sulfonamide (5 g, 0.0228 mol, see Example 1(a) above) was dissolved in THF (43 mL) under N₂ and then cooled to -78°C. *n*-BuLi (1.6 M, 38.5 mL, 0.062 mol) was added *via* a syringe. The reaction mixture was stirred at -78 °C for 30 min and then at -40°C for 2 h. Iodobutane (5.19 mL, 0.046 mol) was added dropwise to the reaction mixture. The reaction mixture was stirred overnight at room temperature, quenched with NH₄Cl (aq) and extracted with EtOAc. The combined organic phase was washed with brine, dried and concentrated *in vacuo*. The crude product was purified on column chromatography (Hex:EtOAc 10:1) to give the sub-

title compound in 46% yield (2.92 g, 0.011 mol).

^1H NMR δ (CDCl_3): 7.41 (d, $J = 3.6$ Hz, 1H), 6.69 (d, $J = 3.8$ Hz, 1H), 4.96 (m, 1H), 2.80 (d, $J = 7.6$ Hz, 2H), 1.65 (m, 2H), 1.37 (m, 2H), 1.26 (s, 9H), 0.92 (t, $J = 7.26$ Hz, 3H)

^{13}C NMR δ (CDCl_3): 153.0, 141.7, 131.9, 124.0, 54.9, 33.4, 29.9, 22.0, 13.6.

5

(b) 5-Butyl-2-(*N*-*tert*-butylaminosulfonyl)thiophene-3-boronic acid

5-Butylthiophene-2-sulfonic acid *tert*-butylamide (2.9 g, 0.010 mol, see step (a) above) was dissolved in THF (40 mL) under N_2 and then cooled to -78°C . *n*-BuLi (1.6 M, 16.2 mL, 0.026 mol) was added *via* a syringe. The
10 reaction mixture was stirred at -20°C for 4 hours. Tri-*iso*-propylborate (13.3 mL, 0.058 mol) was then added *via* a syringe and the reaction mixture was stirred overnight at room temperature. The reaction was quenched with 2 M HCl (20 mL). The organic phase was separated and the water phase was extracted with EtOAc (3 x 100 mL). The combined organic phase was
15 washed with brine and dried and concentrated *in vacuo*. The product was used in the next step without further purification.

MS (ESI^+) m/z : 320.1

20 (c) 3-(4-Imidazol-1-ylmethylphenyl)-5-butyl-*N*-*tert*-butylthiophene-2-sulfonamide

5-Butyl-2-(*N*-*tert*-butylaminosulfonyl)thiophene-3-boronic acid (300 mg, 1.27 mmol, see step (b) above), 1-(4-bromobenzyl)-1H-imidazole (606 mg, 1.90 mmol, see Example 1(d) above), toluene (15 mL), ethanol (4 mL), NaOH (1.0 M, 4.0 mL, 5.1 mmol) and $\text{Pd}(\text{PPh}_3)_4$ (43.9 mg, 0.038 mmol)
25 were mixed under N_2 . The mixture was warmed to reflux for 2 hours, diluted with EtOAc (50 mL), washed with water and brine, and dried over MgSO_4 . The solvent was removed and the residue was separated by column chromatography using chloroform:methanol (20:1) as eluent. The product was not completely pure but was used in the next step without
30 further purification.

(d) 3-(4-Imidazol-1-ylmethylphenyl)-5-butylthiophene-2-sulfonamide

Trifluoroacetic acid was added (10 mL) to the crude 3-(4-imidazol-1-ylmethylphenyl)-5-butyl-*N-tert*-butylthiophene-2-sulfonamide from step (c) above, and one drop (*ca.* 0.05 mL) of anisol was added to the mixture. The reaction mixture was stirred under N₂ atmosphere for 30 hours and then evaporated and co-evaporated with acetonitrile. The crude product was purified by column chromatography (CH₂Cl₂:MeOH (20:1)) to give sub-
10 title compound (232 mg, 0.62 mmol) in 49% yield (from 1-(4-bromobenzyl)-1H-imidazole).

¹H NMR δ(CDCl₃, CD₃OD): 8.97(1H,s), 7.64-7.40 (m, 6H), 6.82 (s, 1H), 5.44 (s, 2H), 2.83 (t, *J* = 7.6 Hz, 2H), 1.68 (m, 1H), 1.39 (m, 1H), 0.95 (t, *J* = 7.26 Hz, 1H)

¹³C NMR δ(CDCl₃, CD₃OD): 150.3, 143.8, 136.9, 136.2, 134.9, 131.1,

129.7, 129.3, 123.1, 121.5, 53.3, 34.4, 30.3, 22.9, 14.0

MS (ESI⁺) *m/z*: 376.1

(e) *N*-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-butylthiophene-2-sulfonamide

3-(4-Imidazol-1-ylmethylphenyl)-5-butylthiophene-2-sulfonamide (232 mg, 0.62 mmol; see step (d) above) was dissolved in pyridine (3 mL, dried over 4Å molecular sieves). Pyrrolidinopyridine (91.6 mg, 0.618 mmol) and butyl chloroformate (785.7 μL, 0.618 mmol) were added to the mixture. The mixture was stirred overnight under a N₂ atmosphere at room
25 temperature. Evaporation and co-evaporation with acetonitrile to remove the solvents, followed by purification by column chromatography using 10% MeOH in chloroform as eluent, gave the title compound (29 mg, 0.061 mmol) in 10% yield.

¹H NMR δ(CD₃OD): 7.94 (s, 1H), 7.63 (d, *J* = 8.1 Hz, 2H), 7.28 (d, *J* = 8.1 Hz, 2H), 7.20 (s, 1H), 7.06 (s, 1H), 6.8 (s, 1H), 5.25 (s, 2H), 3.88 (t, *J* = 6.3

Hz, 2H), 2.84 (t, $J = 7.4$ Hz, 2H), 1.68 (m, 1H), 1.44 (m, 4H), 1.26 (m, 2H), 0.96 (t, $J = 7.3$ Hz, 3H), 0.87 (t, $J = 7.3$ Hz, 3H)

^{13}C NMR $\delta(\text{CD}_3\text{OD})$: 159.5, 150.2, 143.9, 138.4, 137.2, 136.6, 131.0, 129.5, 128.5, 128.0, 121.4, 66.5, 51.7, 49.0, 34.7, 32.1, 30.5, 23.2, 20.1,

14.1

MS (ESI⁺) m/z : 476.1

Example 6

N-Butyloxycarbonyl-2-(4-imidazol-1-ylmethylphenyl)-4-*iso*-butylbenzene-sulfonamide

(a) *N*-tert-Butyl-4-*iso*-butylbenzenesulfonamide

Chlorosulfonic acid (28.6 mL, 0.43 mol) was added dropwise to stirred *iso*-butylbenzene (11.14 g, 0.083 mol) at 0°C. The reaction mixture was then heated to 40°C for 0.5 h, poured into ice-water (150 mL) and extracted with ethyl acetate (400 mL). The organic phase was washed with water and brine and dried over MgSO_4 . The solvent was removed *in vacuo* and the residue was dissolved in CHCl_3 (50 mL). To this stirred solution, *tert*-butylamine (43.7 mL, 0.416 mol) was added dropwise. The reaction was heated to reflux for 10 min and then cooled to room temperature. The reaction mixture was then diluted with toluene (200 mL) and washed with water and brine. The organic phase was dried over MgSO_4 and the solvent was removed *in vacuo*. Purification using column chromatography with hexane:acetone (4:1) as eluent yielded the sub-title compound as a white solid (12.0 mg, 0.045 mol) in 54% yield.

IR (neat, cm^{-1}) ν 3266, 2960, 2925, 2871, 1597, 1455

^1H NMR $\delta(\text{CDCl}_3)$: 7.84 (d, $J = 8.4$ Hz, 2H), 7.27 (d, $J = 8.3$ Hz, 2H), 5.11 (brs, 1H), 2.55 (d, $J = 7.3$ Hz, 2H), 1.90 (m, 1H), 1.65 (m, 2H), 1.21 (s, 9H), 0.92 (d, $J = 6.6$ Hz, 6H)

^{13}C NMR $\delta(\text{CDCl}_3)$: 146.4, 140.7, 129.9, 129.5, 126.8, 54.5, 45.1, 30.1, 22.3

MS (ESI⁺) m/z : 270.0

Anal. Calcd for $\text{C}_{14}\text{H}_{23}\text{NO}_2\text{S}$: C, 62.42; H, 8.61; N, 5.20; O, 11.88; S, 11.90;

5 Found: C, 62.2; H, 8.5; N, 5.2

(b) 4-*iso*-Butyl-2-(*N*-*tert*-butylaminosulfonyl)benzene-3-boronic acid

To a solution of *N*-*tert*-butyl-4-*iso*-butyl-benzenesulfonamide (2.69 g, 10 mmol, see step (a)) in THF (50 mL), *n*-BuLi (15.6 mL, 1.6M, 25 mmol) was added dropwise at -78°C under an atmosphere of N_2 (g). The temperature was allowed to rise gradually to 0°C over 2 h and was then kept at that temperature for 30 min. The reaction mixture was then cooled to -40°C and tri-*iso*-propylborate (4.6 mL, 20 mmol) was added. The reaction mixture was stirred overnight at ambient temperature and was quenched with 2M HCl (20 mL). The organic phase was separated and the water phase was extracted with EtOAc (3 x 100 mL). The combined organic phase was washed with brine and dried and concentrated *in vacuo*. The crude product was used in the next step without further purification.

MS (ESI⁺) m/z : 314.0

(c) 2-(4-Imidazol-1-ylmethylphenyl)-4-*iso*-butyl-*N*-*tert*-butylbenzene-sulfonamide

The crude product from step (b) above (1.2 g, 3.83 mmol), 1-(4-bromobenzyl)-1H-imidazole (98.8 mg, 0.416 mmol, see Example 1(d) above), Pd(PPh₃)₄ (29 mg, 0.25 mmol), NaOH (3 mL, 1M, 3 mmol), toluene (15 mL) and ethanol (3 mL) were mixed under N_2 (g). The mixture was heated to reflux for 2 h. The reaction mixture was then diluted with ethyl acetate (150 mL) and washed with water and brine. The organic phase was dried over MgSO_4 and the solvent was removed *in vacuo*. Purification using

column chromatography with CHCl_3 : MeOH (20:1) as eluent yielded the sub-title compound (93.7 mg, 0.220 mmol) in 53% yield.

IR (neat, cm^{-1}) ν 3379, 3293, 3153, 2955, 2868, 1701, 1596, 1505

^1H NMR $\delta(\text{CDCl}_3)$: 8.05 (d, $J = 8.1$ Hz, 1H), 7.80 (s, 1H), 7.53 (d, $J = 8.2$ Hz, 2H), 7.30 (m, 3H), 7.07 (m, 3H), 5.24 (s, 2H), 2.56 (d, $J = 7.1$ Hz, 2H), 1.90 (m, 1H), 1.01 (s, 9H), 0.93 (d, $J = 6.6$ Hz, 6H)

^{13}C NMR $\delta(\text{CDCl}_3)$: 146.2, 139.9, 138.9, 138.8, 136.7, 135.5, 132.8, 130.3, 128.4, 128.2, 127.9, 126.6, 119.5, 54.0, 50.6, 44.7, 29.8, 29.4, 22.0

MS (ESI^+) m/z : 426.1

10

(d) 2-(4-Imidazol-1-ylmethylphenyl)-4-iso-butylbenzene-sulfonamide

To a solution of 2-(4-imidazol-1-ylmethylphenyl)-4-iso-butyl-*N*-tert-butylbenzene-sulfonamide (0.211 mmol, 90.0 mg, see step (c) above) in CH_2Cl_2 (5 mL) was added BCl_3 (1.5 mL, 1M, 1.5 mmol) under N_2 (g). The mixture was stirred for 0.5 h. Water (50 mL) was added and the mixture was extracted with ethyl acetate (3 x 50 mL). The combined organic phases were washed with brine and dried over MgSO_4 and the solvent was removed *in vacuo*. The crude product was used directly in the next step without further purification.

20

(e) *N*-Butyloxycarbonyl-2-(4-imidazol-1-ylmethylphenyl)-4-iso-butylbenzenesulfonamide

The crude product from step (d) above was dissolved in pyridine (2 mL, dried over 4 Å molecular sieve). Pyrrolidinopyridine (36 mg, 0.024 mmol) and butyl chloroformate (276 μL , 2.23 mmol) were added to the mixture, which was then stirred for 30 h under N_2 (g) at room temperature. The solvent was removed *in vacuo* and then co-evaporated with acetonitrile. Purification using column chromatography with CHCl_3 : MeOH (10:1) as eluent yielded the title compound (66.7 mg, 0.142 mmol) in 68% yield

(from 2-(4-imidazol-1-ylmethylphenyl)-4-*iso*-butyl-*N*-*tert*-butylbenzene-sulfonamide).

IR (neat, cm^{-1}) ν 3129, 3058, 2956, 2869, 1737, 1658, 1466

^1H NMR δ (CDCl_3 , CH_3OD): 8.14 (d, J = 8.1 Hz, 1H), 7.72 (s, 1H), 7.36-7.19 (m, 6H), 7.04 (m, 3H), 5.19 (s, 2H), 3.98 (t, J = 6.5 Hz, 2H), 2.56 (d, J = 7.1 Hz, 2H), 1.93 (m, 1H), 1.41 (m, 2H), 1.21 (m, 2H), 0.93 (d, J = 6.6 Hz, 6H), 0.85 (t, J = 7.1 Hz, 3H)

^{13}C NMR δ (CDCl_3 , CH_3OD): 151.8, 147.7, 140.4, 139.6, 137.1, 135.3, 134.9, 133.2, 130.5, 129.9, 128.6, 128.0, 127.0, 119.9, 66.3, 45.1, 30.6, 30.1, 22.4, 18.9, 13.6

MS (ESI^+) m/z : 470.1

Example 7

N-Butyloxycarbonyl-5-*iso*-butyl-3-(4-tetrazol-2-ylmethylphenyl)thiophene-2-sulfonamide

(a) 3-(4-Hydroxymethylphenyl)-5-*iso*-butyl-*N*-*tert*-butylthiophene-2-sulfonamide

5-*iso*-Butyl-2-(*N*-*tert*-butylaminosulfonyl)thiophene-3-boronic acid (319.3 mg, 1.00 mmol, see Example 1(c) above), 4-bromobenzyl alcohol (374.1 mg, 2.00 mmol), toluene (20 mL), ethanol (4 mL), NaOH (1.0M, 4 mL, 4 mmol) and $\text{Pd}(\text{PPh}_3)_4$ (34 mg, 0.030 mmol) were mixed together under N_2 . The mixture was warmed to reflux for 2 hours and was then diluted with EtOAc (50 mL), washed with water and brine and dried over MgSO_4 . The solvent was removed and the residue was separated by column chromatography using CHCl_3 :MeOH (40:1) as eluent to give 289 mg of the sub-title compound (yield: 76%).

IR(neat): 3465, 3162, 2952, 2867, 1441 cm^{-1}

^1H NMR $\delta(\text{CD}_3\text{OD})$: 7.59(2H, d, $J = 8.2$ Hz), 7.45(2H, d, $J = 8.2$ Hz), 6.75(1H, s), 4.75(2H, s), 4.11(1H, brs), 2.69(2H, d, $J = 7.1$ Hz), 1.92(1H, m), 0.99(6H, d, $J = 7.2$ Hz), 0.98(9H, s)

^{13}C NMR $\delta(\text{CD}_3\text{OD})$: 148.3, 142.9, 141.1, 134.2, 130.3, 128.9, 127.6, 126.8, 64.8, 54.5, 39.2, 30.5, 29.5, 22.1

MS(EI^+) m/z : 382.0

Anal. Calcd for $\text{C}_{19}\text{H}_{27}\text{NO}_3\text{S}_2$: C, 59.8; H, 7.3; N, 3.7. Found: C, 59.6; H, 7.0; N, 3.5

10 (b) 3-(4-Bromomethylphenyl)-5-iso-butyl-N-tert-butylthiophene-2-sulfonamide

3-(4-Hydroxymethylphenyl)-5-iso-butyl-N-tert-butylthiophene-2-sulfonamide (280 mg, 0.734 mmol, see step (a) above) was dissolved in DMF (10 mL). PPh_3 (459.2 mg, 1.75 mmol) and CBr_4 (580.3, 1.75 mmol) were added to the resultant solution. The mixture was stirred for 24 h at room temperature and then diluted with ethyl acetate. The organic phase was washed with water (50 mL) and brine (50 mL) and then dried over MgSO_4 . After removing the solvents, the residue was purified by column chromatography using hexane:acetone (5:1) as eluent to give the sub-title compound (314.9 mg, 0.709 mmol, 76% yield).

IR(neat): 3302, 2952, 2866, 1442 cm^{-1}

^1H NMR $\delta(\text{CDCl}_3)$: 7.62(2H, d, $J = 8.4$ Hz), 7.48(2H, d, $J = 8.4$ Hz), 6.75(1H, s), 4.56(2H, s), 4.11(1H, brs), 2.69(2H, d, $J = 7.1$ Hz), 1.92(1H, m), 0.99(6H, d, $J = 7.2$ Hz), 0.98(9H, s)

25 ^{13}C NMR $\delta(\text{CDCl}_3)$: 148.5, 142.4, 138.2, 136.9, 135.1, 129.5, 129.1, 128.7, 54.6, 39.2, 32.8, 30.5, 29.5, 22.1

MS(EI^+) m/z : 445.8

(c) 5-iso-Butyl-N-tert-butyl-3-(4-tetrazol-2-ylmethylphenyl)thiophene-2-sulfonamide

KOH (112.2 mg, 2.00 mmol, crushed pellets) was added to DMSO (10 mL, dried over 4A molecular sieve) and stirred for 5 min. Tetrazole (28.0 mg, 0.4 mmol) was added to the mixture, which was then stirred for 2 h. 3-(4-Bromomethylphenyl)-5-iso-butyl-N-tert-butylthiophene-2-sulfonamide (130 mg, 0.292 mmol, see step (b) above) was added, the mixture was cooled briefly and stirred for an additional hour before water (50 mL) was added. The reaction mixture was extracted with ethyl acetate (250 mL) and the extract was washed with water (2 x 50 mL) and brine (50 mL). The organic phase was dried over MgSO_4 and the solvent was removed *in vacuo*. The residue was purified on column chromatography using hexane:acetone (3:1) as eluent to give the sub-title compound (28.6 mg, 0.066 mmol, 23% yield). IR(neat): 3328, 3134, 2980, 1501, 1466 cm^{-1}

^1H NMR $\delta(\text{CDCl}_3)$: 8.52(1H, s), 7.64(2H, d, $J = 8.3$ Hz), 7.46(2H, d, $J = 8.3$ Hz), 6.73(1H, s), 5.85(2H, s), 2.69(2H, d, $J = 7.1$ Hz), 1.91(1H, m), 1.58(1H, s), 0.98(15H, brs)

^{13}C NMR $\delta(\text{CDCl}_3)$: 153.2, 148.5, 142.4, 136.8, 135.8, 133.2, 129.7, 128.8, 128.5, 56.3, 54.6, 39.2, 30.5, 29.5, 22.1

MS(EI^+) m/z : 434.0

Anal. Calcd for $\text{C}_{20}\text{H}_{27}\text{N}_5\text{O}_2\text{S}_2 \times \text{H}_2\text{O}$: C, 53.2; H, 6.5; N, 15.5. Found: C, 53.7; H, 6.1; N, 15.2

(d) 5-iso-Butyl-3-(4-tetrazol-2-ylmethylphenyl)thiophene-2-sulfonamide

To a solution of 5-iso-butyl-N-tert-butyl-3-(4-tetrazol-2-ylmethylphenyl)-thiophene-2-sulfonamide (42.1 mg, 0.111 mmol, see step (c) above) in CH_2Cl_2 (10 mL) was added BCl_3 (0.5 mL, 1M, 0.5 mmol) under N_2 (g). The reaction mixture was stirred for 0.5 h. Water (50 mL) was added and the mixture was extracted with ethyl acetate (3 x 50 mL). The combined organic phases were washed with brine and dried over MgSO_4 and the

solvent was removed *in vacuo*. The crude product was used directly in the next step without further purification.

(e) *N*-Butyloxycarbonyl-5-*iso*-butyl-3-(4-tetrazol-2-ylmethylphenyl)thiophene-2-sulfonamide

- The crude product from step (d) above was dissolved in pyridine (1 mL, dried over 4Å molecular sieve). Pyrrolidinopyridine (14 mg, 0.0095 mmol) and butyl chloroformate (120 µL, 0.97 mmol) were added to the mixture, which was then stirred for 30 hours under N₂(g) at room temperature. The solvent was removed *in vacuo* and then co-evaporated with acetonitrile. Purification using column chromatography with CHCl₃:MeOH (35:1) as eluent yielded the title compound (24.9 mg, 0.052 mmol) in 54% yield (from 5-*iso*-butyl-*N*-*tert*-butyl-3-(4-tetrazol-2-ylmethylphenyl)thiophene-2-sulfonamide).
- IR(neat): 3330, 2961, 2875, 1743, 1466 cm⁻¹
- ¹H NMR δ(CDCl₃): 8.49(1H, s), 7.68(1H, s), 7.48(2H, d, J= 8.2 Hz), 7.40(2H, d, J=8.2 Hz), 6.73(1H, s), 5.82(2H, s), 4.07(2H, t, J= 6.6 Hz), 2.70(2H, d, J= 7.1 Hz), 1.91(1H, m), 1.50(2H, m), 1.24(2H, m), 0.98(6H, d, J= 6.9 Hz), 0.87(3H, J= 7.4 Hz)
- ¹³C NMR δ(CDCl₃): 153.2, 151.8, 150.1, 145.6, 134.8, 133.4, 129.6, 129.3, 128.3, 66.9, 56.3, 39.2, 30.5, 30.4, 22.2, 18.7, 13.6
- MS(EI⁺) m/z: 478.0
- Anal. Calcd for C₂₁H₂₇N₅O₄S₂: C, 52.8; H, 5.7; N, 14.7. Found: C, 53.0; H, 5.8; N, 14.1

Example 8N-Butyloxycarbonyl-5-iso-butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-2-sulfonamide5 (a) 1-(4-Bromobenzyl)-1H-tetrazole

Dimethyl sulphoxide (10 mL, dried over 4A molecular sieve) was added to potassium hydroxide (1.12 g, 0.02 mol, crushed pellets) and the mixture was stirred for 5 minutes. 1H-Tetrazole (0.35 g, 0.005 mol) was then added and the mixture was stirred for 2 hours. 4-Bromobenzyl bromide (1.87 g, 0.0075 mol) was added and the mixture was cooled briefly and stirred for a further hour before adding water (50 mL). The mixture was extracted with ether (3 × 80 mL) and each extract was washed with water (3 × 50 mL). The combined ether layers were dried over MgSO₄ and the solvent using removed *in vacuo*. The residue was chromatographed on silica gel with CHCl₃:MeOH (40:1) as eluent yielding the sub-title compound (0.98 g, yield: 82%).

¹H NMR δ(CDCl₃): 8.64(1H, s), 7.50(2H, d, J= 8.4 Hz), 7.18(2H, d, J= 8.4), 5.56(2H, s)

¹³C NMR δ(CDCl₃): 142.4, 132.4, 131.8, 129.9, 123.4, 51.3

20 MS(ESI⁺) m/z: 238.8

Anal. Calcd. for C₈H₇BrN₄: C, 40.2; H, 3.0; N, 23.4. Found: C, 40.3; H, 3.0; N, 23.4

25 (b) 5-iso-Butyl-N-tert-butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-2-sulfonamide

5-iso-Butyl-2-(N-tert-butylaminosulfonyl)thiophene-3-boronic acid (401.0 mg, 1.256 mmol, see Example 1(c) above), 1-(4-bromobenzyl)-1H-tetrazole (199.4 mg, 0.834 mmol, see step (a) above), toluene (20 mL), ethanol (3.0 mL), NaOH (1.0M, 5.0 mL, 5.0 mmol) and Pd(PPh₃)₄ (29.0 mg, 0.25 mmol) were mixed under N₂. The mixture was warmed to reflux for 2 hours. The

mixture was diluted with EtOAc (20 mL), washed with water and brine, and dried over MgSO₄. The solvent was removed and the residue was separated by column chromatography using CHCl₃:MeOH (40:1) as eluent to give 222.4 mg of the sub-title compound (yield: 62%).

5 IR(neat): 3284, 3134, 2958, 2870, 1513, 1436 cm⁻¹

¹H NMR δ(CDCl₃): 8.71(1H, s), 7.64(2H, d, J= 8.3 Hz), 7.40(2H, d, J= 8.3 Hz), 6.74(1H, s), 5.65(2H, s), 2.67(2H, d, J= 7.1 Hz), 1.94(1H, m), 0.99(15H, m)

¹³C NMR δ(CDCl₃): 148.5, 142.6, 142.2, 136.8, 135.9, 133.1, 129.9, 128.8,
10 128.3, 54.6, 51.7, 39.2, 30.5, 29.5, 22.1

MS(ESI⁺) m/z: 433

(c) 5-iso-Butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-2-sulfonamide

BCl₃ (1.0 mL, 1M, 1.0 mmol) was added to a solution of 5-iso-butyl-*N*-tert-butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-2-sulfonamide (177.0 mg, 0.408 mmol, see step (b) above) in CH₂Cl₂ (10 mL) under N₂ (g), and the reaction mixture was stirred for 0.5 h. Water (50 mL) was added and the mixture was extracted with ethyl acetate (3 x 50 mL). The combined organic phases were washed with brine, dried over MgSO₄ and the solvent
20 was removed *in vacuo*. The crude product was used directly in the next step used without further purification.

(d) *N*-Butyloxycarbonyl-5-iso-butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-2-sulfonamide

25 The title compound was prepared (89.6 mg, 0.188 mmol, 46% yield (from 5-iso-butyl-*N*-tert-butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-2-sulfonamide)) analogously to the procedure described in Example 7(e) above from the crude 5-iso-butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-2-sulfonamide from step (c) above.

30 IR(neat): 3135, 2959, 2875, 1747, 1464 cm⁻¹

^1H NMR δ (CDCl_3): 8.73(1H, s), 7.43(2H, d, $J=7.7$ Hz), 7.24(2H, d, $J=7.7$ Hz), 6.72(1H, s), 5.59(2H, s), 4.00(2H, brs), 2.69(2H, brs), 1.91(1H, m), 1.46(2H, m), 1.19(2H, m), 0.95(6H, d, $J=6.9$ Hz), 0.83(3H, $J=6.8$ Hz)

^{13}C NMR δ (CDCl_3): 151.8, 151.4, 145.3, 143.0, 134.8, 133.5, 129.6, 129.1,

5 127.8, 66.9, 51.4, 39.2, 30.9, 30.4, 22.2, 18.7, 13.6

MS(EI^+) m/z : 478.0

Anal. Calcd for $\text{C}_{21}\text{H}_{27}\text{N}_5\text{O}_4\text{S}_2 \times \frac{1}{2} \text{H}_2\text{O}$: C, 51.8; H, 5.8; N, 14.4. Found: C, 51.4; H, 5.6; N, 14.1

10 Example 9

N-Butyloxycarbonyl-3-(4-[1,2,4]triazol-1-ylmethylphenyl)-5-*iso*-butyl-thiophene-2-sulfonamide

(a) 1-(4-Bromobenzyl)-1*H*-[1,2,4]triazole

15 DMF and KOH (3.3 g, 58 mmol) were stirred together at rt for 5 minutes before adding 1,2,4-triazole (1 g, 14.5 mmol). After a further 30 minutes, the reaction mixture was cooled to 0°C and 1-bromo-4-bromomethylbenzene (7.2 g, 29 mmol) was added dropwise over 5 minutes. The reaction mixture was heated to 60°C, then cooled to rt, extracted with ethyl acetate
20 and water, and subsequently dried over K_2CO_3 . The solvent was evaporated to yield yellow-white crystals, which, upon repeated recrystallisation, (ethylacetate/isohexane) yielded 0.60 g of the sub-title compound as white crystals (62% isolated yield).

^1H NMR δ (270 MHz, CDCl_3): 8.11 (s, 1H), 7.96 (s, 1H), 7.51-7.38 (m, 2H),
25 7.15-7.10 (m, 2H), 5.29 (s, 2H)

^{13}C NMR δ (67.8 MHz, CDCl_3): 152.2, 143.0, 133.5, 132.1, 129.5, 122.7, 52.8

MS m/z 238 ($\text{M}^+ + 1$)

(b) 3-(4-[1,2,4]Triazol-1-ylmethylphenyl)-5-iso-butyl-N-tert-butylthio-
phene-2-sulfonamide

5-iso-Butyl-2-(N-tert-butylaminosulfonyl)thiophene-3-boronic acid (0.479 g, 1.5 mmol, see Example 1(c) above), 1-(4-bromobenzyl)-1H-[1,2,4]triazole (0.238 g, 1 mmol, see step (a) above), Pd(OAc)₂ (15.7 mg, 0.03 mmol), triphenyl phosphine (15.7 mg, 0.06 mmol) and NaOH (0.16 g, 4 mmol) were dissolved in 4 mL of toluene/ethanol (4:1) in a thick walled glass tube, and were then heated to 80°C for 1 h. The reaction mixture was cooled to rt, extracted with ethyl acetate and water and subsequently dried over K₂CO₃. The solvent was evaporated and the reaction mixture was separated on a silica column (dichloromethane + 1% methanol to dichloromethane + 4% methanol) to yield 0.288 g of the sub-title compound (65% yield).

¹H NMR δ(270 MHz, CDCl₃): 8.13 (s, 1H), 7.94 (s, 1H), 7.60-7.57 (m, 2H), 7.33-7.30 (m, 2H), 6.72 (s, 1H), 5.37 (s, 2H), 4.47 (s, 1H), 2.65, (d, J = 7 Hz, 2H), 1.89 (sept J = 7 Hz, 1H), 0.96 (s, 9H), 0.94 (d, J = 7 Hz, 6H)

¹³C NMR δ(67.8 MHz, CDCl₃): 152.1, 148.5, 143.1, 142.3, 136.6, 135.2, 134.8, 129.6, 128.8, 128.0, 54.5, 53.1, 39.1, 30.4, 29.4, 22.1

MS m/z 433 (M⁺ + 1)

(c) 3-(4-[1,2,4]Triazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

3-(4-[1,2,4]Triazol-1-ylmethylphenyl)-5-iso-butyl-N-tert-butylthiophene-2-sulfonamide (146.4 mg, 0.34 mmol, see step (b) above) was mixed with BCl₃ (1M solution in hexane) (2 mL, 1.7 mmol) in 5 mL of dichloromethane at rt and stirred for 1 h. The reaction mixture was extracted with ethyl acetate and water and subsequently dried over K₂CO₃. The solvent was evaporated and the resultant product was sufficiently pure to be used directly in the next step.

(d) N-Butyloxycarbonyl-3-(4-[1,2,4]triazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

59 mg (0.16 mmol) of the crude 3-(4-[1,2,4]triazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide from step (c) above was mixed with butyl
5 chloroformate (31 μ L, 0.24 mmol) and 4-dimethylaminopyridine (2 mg, 16 μ mol) in 5 mL of triethylamine at 0°C. The reaction mixture was stirred overnight and then diluted with ethyl acetate, washed with water and dried over K_2CO_3 . The reaction mixture was then separated on a silica column (dichloromethane + 15% methanol), circular chromatography
10 (dichloromethane + 10-15% methanol and preparative LC-MS to yield 7.0 mg of the title compound (9% isolated yield).

1H NMR δ (270 MHz, $CDCl_3$): 8.11 (s, 1H), 7.98 (s, 1H), 7.50-7.47 (m, 2H), 7.29-7.26 (m, 2H), 6.74 (s, 1H), 5.39 (s, 2H), 4.05 (t, $J = 7$ Hz, 2H), 2.71 (d, $J = 7$ Hz, 2H), 1.95 (sept, $J = 7$ Hz, 1H), 1.52 (pent, $J = 7$ Hz, 2H), 1.26
15 (sext, $J = 7$ Hz, 2H), 0.99 (d, $J = 7$ Hz, 6H), 0.88 (t, $J = 7$ Hz, 3H)

^{13}C NMR δ (67.8 MHz, $CDCl_3$): 151.9, 151.1, 145.1, 143.4, 134.7, 134.2, 131.6, 129.7, 129.0, 127.8, 66.3, 53.2, 39.2, 30.4, 30.3, 22.1, 18.7, 13.5

MS m/z (relative intensity 30 eV) 477 ($M^+ + 1$)

20 Example 10

N-(Butylamino)carbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

(a) 1-(4-Bromobenzyl)-1H-imidazole
25 Dimethyl sulphoxide (20 mL, dried over 4Å molecular sieves) was added to potassium hydroxide (2.24 g, 0.04 mol, crushed pellets) and the mixture was stirred for 5 min. Imidazole (0.5718 g, 0.0084 mol) was then added and the mixture was stirred for 2 hours. 4-Bromobenzyl bromide (3.25 g, 0.013 mol) was added and the mixture was cooled briefly and stirred for a
30 further hour before adding water (20 mL). The mixture was extracted with

ether (3 × 100 mL) and each extract was washed with water (3 × 50 mL). The combined ether layers were dried over CaCl₂ and the solvent was removed *in vacuo*. The residue was chromatographed on silica gel with CHCl₃/MeOH (30:1) plus 0.05% formic acid as eluent yielding the sub-title compound (1.275 g, yield: 53%).

¹H NMR δ(CDCl₃): 7.73 (m, 3H), 7.28 (m, 3H), 7.15 (m, 1H), 5.30 (s, 2H)

¹³C NMR δ(CDCl₃): 136.8, 134.8, 131.5, 129.3, 128.4, 121.5, 118.7, 49.4

MS (ESI⁺) *m/z*: 236.8

(b) 3-(4-Imidazol-1-ylmethylphenyl)-5-iso-butyl-N-tert-butylthiophene-2-sulfonamide

5-iso-Butyl-2-(N-tert-butylaminosulfonyl)thiophene-3-boronic acid (200.5 mg, 0.628 mmol, see Example 1(c) above), 1-(4-bromobenzyl)-1H-imidazole (98.8 mg, 0.416 mmol, see step (a) above), toluene (15 mL), ethanol (15 mL), NaOH (1.0M, 1.5 mL, 1.5 mmol) and Pd(PPh₃)₄ (14.5 mg, 0.125 mmol) were mixed under N₂. The mixture was warmed to reflux for 2 hours. The mixture was diluted with EtOAc (50 mL), washed with water and brine, and dried over MgSO₄. The solvent was removed and the residue was separated by column chromatography with chloroform:methanol (20:1) as eluent to give 113.9 mg of the sub-title compound (yield: 63.27%).

IR (neat, cm⁻¹) ν 3060, 2996, 1507

¹H NMR δ(CDCl₃): 7.39 (s, 1H), 7.35 (d, *J* = 8.1 Hz, 2H), 6.98 (d, *J* = 8.1 Hz, 2H), 6.96 (s, 1H), 6.84 (s, 1H), 6.47 (s, 1H), 4.91 (s, 2H), 3.96 (s, 1H), 2.72 (brs, 1H), 2.42 (d, *J* = 7.1 Hz, 2H), 1.64 (m, 1H), 0.73 (s, 9H), 0.72 (d, *J* = 6.9 Hz, 6H)

¹³C NMR δ(CDCl₃): 148.6, 142.3, 137.2, 136.2, 135.1, 129.7, 129.4, 128.8, 127.4, 119.2, 54.6, 50.6, 39.2, 30.5, 29.5, 22.1

MS (ESI⁺) *m/z*: 431.9

Anal. Calcd for C₂₂H₂₉N₃O₂S₂: C, 58.8; H, 7.0; N, 9.4. Found: C, 58.7.0; H, 6.7; N, 9.1

(c) 3-(4-Imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

To a solution of 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butyl-N-*tert*-butylthiophene-2-sulfonamide (0.097 mmol, 42.0 mg, see step (b) above) in CH₂Cl₂ (10 mL) was added BCl₃ (0.5 mL, 1M, 0.5 mmol) under N₂ (g). The mixture was stirred for 0.5 h. Water (50 mL) was added and the mixture was extracted with ethyl acetate (3 x 50 mL). The combined organic phases were washed with brine and dried over MgSO₄ and the solvent was removed *in vacuo*. The crude product was used directly in the next step without further purification.

(d) N-(Butylamino)carbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

The crude product from step (c) above was dissolved in acetone (5 mL) under N₂ (g). NaOH (0.20 mL, 1M, 0.20 mmol) was added to the mixture, which was then stirred for 10 min. Butyl isocyanate (109 μ L, 0.97 mmol) was then added and the mixture was stirred overnight at room temperature. The reaction mixture was then diluted with ethyl acetate (150 mL) and washed with water and brine. The organic phase was dried over MgSO₄ and the solvent was removed *in vacuo*. Purification using column chromatography with CHCl₃:MeOH (10:1) as eluent yielded the title compound (15.1 mg, 0.032 mmol) in 33% yield (from 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonic acid *tert*-butylamide).

IR (neat, cm⁻¹) ν 3261, 3120, 2957, 2869, 1701, 1514

¹H NMR δ (CDCl₃, CH₃OD): 7.64 (s, 1H), 7.49 (d, *J* = 8.1 Hz, 2H), 7.11 (d, *J* = 8.1 Hz, 2H), 6.97 (brs 1H), 6.90 (brs, 1), 6.72 (s, 1H), 6.24 (brs, 1H), 5.10 (s, 2H), 3.08 (m, 2H), 2.62 (d, *J* = 7.1 Hz, 2H), 1.92 (m, 1H), 1.20 (m, 4H), 0.99 (d, *J* = 6.6, 6H), 0.86 (t, *J* = 7.1 Hz, 3H)

^{13}C NMR $\delta(\text{CDCl}_3, \text{CH}_3\text{OD})$: 152.2, 150.0, 144.5, 137.0, 135.9, 134.4, 133.0, 129.7, 129.5, 128.1, 127.1, 119.5, 50.7, 39.9, 39.2, 31.6, 30.5, 22.2, 19.8, 13.7

MS (ESI^+) m/z : 475.2

5

Example 11

N-Butylsulfonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide

Crude 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-sulfonamide (prepared according to the procedure described in Example 10(c) above) was dissolved in THF (3 mL) under N_2 (g). NaOH (1.0 mL, 1M, 1.0 mmol) was added to the mixture, which was then stirred for 10 min. Butanesulfonyl chloride (45 μL , 0.35 mmol) was then added, and the mixture was stirred for 24 h at room temperature. The reaction mixture was then diluted with ethyl acetate (150 mL) and washed with water and brine. The organic phase was dried over MgSO_4 and the solvent was removed *in vacuo*. The crude product was recrystallised from acetone to yield the title compound (31.7 mg, 0.064 mmol).

IR (neat, cm^{-1}) ν 3133, 2959, 2871, 1576, 1543, 1514

^1H NMR $\delta(\text{CDCl}_3, \text{CH}_3\text{OD})$: 8.70 (s, 1H), 7.64 (d, $J = 8.1$ Hz, 2H), 7.08-7.20 (m, 5H), 6.59 (s, 1H), 5.06 (s, 2H), 3.08 (m, 2H), 2.57 (d, $J = 7.1$ Hz, 2H), 1.67 (m, 1H), 1.65 (m, 2H), 1.29 (m, 2H), 0.89-0.79 (m, 9H)

^{13}C NMR $\delta(\text{CDCl}_3, \text{CH}_3\text{OD})$: 146.8, 140.9, 138.2, 136.8, 135.0, 131.9, 130.4, 128.6, 127.9, 121.2, 119.8, 54.0, 52.5, 38.9, 30.3, 25.6, 21.9, 21.4, 13.4

25

MS (ESI^+) m/z : 496.1

Example 12N-Butylsulfonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-carboxamide5 (a) 2-iso-Butylthiophene

A solution of thiophene (6.00 g, 0.0714 mol) in 80 mL of THF (80 mL) was treated with *n*-BuLi (1.6 M in hexane, 54 mL, 0.0864 mol) at -78°C. The mixture was stirred at -40°C for about 2 hours. The solution was then cooled to -78°C again and treated with 2-methylpropyliodide (16.04 g, 0.0871 mol). The solution was stirred at 0°C for 2 hours, and then overnight at room temperature for about 16 hours. The solution was treated with water (25 mL) and extracted with petroleum ether (3 x 25 mL). The organic layer was dried over magnesium sulfate and filtered. The crude product was purified by distillation (54-55°C at 12 mmHg) to give the sub-

10 title compound (3.0 g, 0.0213 mol) in 30% yield.

¹H NMR δ(CDCl₃): 7.14 (dd, *J* = 5.1, 1.2 Hz, 1H), 6.95 (dd, *J* = 5.1, 3.3 Hz, 1H), 6.79 (dd, *J* = 3.3, 1.2 Hz, 1H), 2.72 (d, *J* = 7.1 Hz, 2H), 1.92 (m, 1H), 0.97 (d, *J* = 6.7 Hz, 3H)

¹³C NMR δ(CDCl₃): 144.3, 126.5, 124.8, 123.0, 39.1, 30.8, 22.2

20 MS(EI⁺) *m/z*: 140

(b) 5-iso-Butyl-N-tert-butylthiophene-2-carboxamide

To 2-iso-butylthiophene (1 g, 7.143 mmol, see step (a) above) in THF (15mL) was added *n*-BuLi (1.6 M in hexane, 5.3 mL, 8.48 mmol) at -78°C and the reaction mixture was stirred at 0°C for 2 hours. *tert*-Butyl-isocyanate (897 μL, 7.86 mmol) was then added to the mixture at -78°C. The stirred solution was kept at 0°C for an additional 2 hours. The reaction mixture was then quenched with H₂O (15 mL) and extracted with EtOAc (3 x 20 mL). The organic layer was dried over MgSO₄, concentrated and

30 purified using silica gel column chromatography (10:90 petroleum ether-

BtOAc) to give the sub-title compound (1.2 g, 5.01 mmol) as white needles in 70% yield.

¹H NMR δ (CDCl₃): 7.24 (d, J = 3.6 Hz, 1H), 6.69 (d, J = 3.6 Hz, 1H), 5.72 (brs, 1H), 2.65 (d, J = 7.2 Hz, 2H), 1.88 (m, 1H), 1.44 (s, 9H), 0.93 (d, J = 6.6 Hz, 3H)

¹³C NMR δ (CDCl₃): 161.4, 149.2, 137.6, 127.5, 125.5, 51.8, 39.5, 30.7, 28.9, 22.1

IR(neat): 3215, 2924, 1620, 1550, 1464, cm⁻¹

Anal. Calcd. for C₁₃H₂₁NOS: C, 65.2; H, 8.8; N, 5.9. Found: C, 65.5; H, 8.9; N, 5.9.

MS(EI⁺) m/z : 239

(c) 5-iso-Butyl-*N*-tert-butylthiophene-2-carboxamide-3-boronic acid

To a solution of 5-iso-butyl-*N*-tert-butylthiophene-2-carboxamide (from step (b) above; 0.5 g, 2.1 mmol) in THF (50 mL) was added 3.3 mL *n*-BuLi (1.6M in hexane, 3.3 mL, 5.28 mmol) at -78°C. The reaction mixture was slowly warmed to -20°C and stirred for 4 hours. Tri-iso-propylborate (0.59 g, 3.14 mmol) was added to the mixture at -78°C. The solution was slowly warmed to room temperature and stirred overnight. The reaction was quenched with HCl(aq) (2M, 2 mL) and extracted with EtOAc (2 x 25 mL), washed with brine, dried with MgSO₄ and concentrated. The crude product was used in the next step without further purification.

MS(EI⁺) m/z : 284

(d) 3-(4-Imidazol-1-ylmethylphenyl)-5-iso-butyl-*N*-tert-butylthiophene-2-carboxamide

5-iso-Butyl-*N*-tert-butylthiophene-2-carboxamide-3-boronic acid (200 mg, 0.706 mmol, see step (c) above), 1-(4-bromobenzyl)-1H-imidazole (80 mg, 0.337 mmol), toluene (5 mL), ethanol (2 mL), NaOH (1.63 M, 0.84 mL, 1.38 mmol) and Pd(PPh₃)₄ (16.3 mg, 0.014 mmol) were mixed together

under N₂. The resultant mixture was warmed to reflux for 2 hours. The mixture was diluted with EtOAc (50 mL), washed with water and brine, and dried over MgSO₄. The solvent was removed and the residue was separated by column chromatography with chloroform:methanol (95:5) as eluent to give 99 mg of the sub-title compound (yield: 74%).

¹H NMR δ (CDCl₃): 7.55 (br s, 1H), 7.41 (d, *J* = 8.1 Hz, 2H), 7.24 (d, *J* = 8.1 Hz, 2H), 7.09 (br s, 1H), 6.89 (br s, 1H), 6.63 (s, 1H), 5.25 (br s, 1H), 5.16 (s, 2H), 2.63 (d, *J* = 6.9 Hz, 2H), 1.89 (m, 1H), 1.13 (s, 9H), 0.95 (d, *J* = 6.6 Hz, 3H)

¹³C NMR δ(CDCl₃): 161.4, 147.4, 140.2, 137.3, 136.3, 136.1, 133.9, 130.0, 129.8, 128.3, 127.6, 119.0, 51.4, 50.4, 39.3, 30.5, 28.4, 22.2

IR(neat): 3113 2930, 1645, 1512 cm⁻¹

MS(EI⁺) *m/z*: 396

Anal. Calcd. for C₂₃H₂₉N₃OS + H₂O: C, 66.8; H, 7.6; N, 10.2. Found: C, 67.0; H, 7.6; N, 9.9.

(e) 3-(4-Imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-carboxamide

Trifluoroacetic acid was added (2.5 mL) to 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butyl-*N-tert*-butylthiophene-2-carboxamide (165 mg, 0.417 mmol, see step (d) above), and one drop (ca. 0.05 mL) of anisol was added to the reaction mixture. The mixture was stirred under a N₂ atmosphere for 30 hours and then evaporated and co-evaporated with acetonitrile. The crude product was purified by column chromatography (CH₂Cl₂:MeOH (85:15)) to give the sub-title compound (117 mg, 0.345 mmol) in 73% yield.

¹H NMR δ(CD₃OD): 9.05 (br s, 1H), 7.63 (br s, 1H), 7.55 (br s, 1H), 7.51 (d, *J* = 8.4 Hz, 2H), 7.45 (d, *J* = 8.4 Hz, 2H), 6.81 (s, 1H), 5.47 (s, 2H), 2.68 (d, *J* = 7.1 Hz, 2H), 1.90 (m, 1H), 0.96 (d, *J* = 6.6 Hz, 3H)

¹³C NMR δ(CD₃OD): 167.1, 148.9, 144.1, 137.9, 136.6, 135.1, 131.2, 130.8, 130.1, 129.8, 123.3, 121.6, 53.4, 40.0, 31.8, 22.5

IR(neat): 3308, 2957, 1657, 1509, 1461

MS(EI⁺) m/z: 340

Anal. Calcd. for C₁₉H₂₁N₃OS: C, 67.2; H, 6.2; N, 12.4. Found: C, 67.5; H, 6.4; N, 12.3.

5

(f) N-Butylsulfonyl-3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-carboxamide

NaH (55%, 12 mg, 0.28 mmol) was added to a solution of 3-(4-imidazol-1-ylmethylphenyl)-5-iso-butylthiophene-2-carboxamide (46 mg, 0.136 mmol),
10 see step (e) above) in THF (1 mL). The mixture was stirred at 50°C for 0.5 hours. Butanesulfonyl chloride (29 µL, 0.223 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 1 hour. The mixture was concentrated and purified using silica gel chromatography (CH₂Cl₂:MeOH, 9:1) to give 31 mg of the title compound as a white powder
15 (yield: 50%).

¹H NMR δ(CDCl₃): 7.82 (br s, 1H), 7.32 (br s, 2H), 7.06 (d, J = 7.4 Hz, 2H), 6.95 (br s, 2H), 6.64 (s, 1H), 5.09 (br s, 2H), 4.79 (br s, 1H), 2.97 (br s, 2H), 2.62 (d, J = 6.9 Hz, 2H), 1.90 (m, 1H), 1.65 (br s, 2H), 1.29 (m, 2H), 0.95 (d, J = 6.5 Hz, 6H), 0.81 (t, J = 6.6 Hz, 3H)

120 ¹³C NMR δ(CDCl₃): 166.4, 148.9, 144.3, 137.3, 136.4, 134.8, 132.2, 130.0, 129.4, 127.2, 119.9, 53.2, 51.0, 39.5, 30.5, 25.4, 22.3, 21.5, 13.6

MS(EI⁺) m/z: 460

Anal. Calcd. for C₂₃H₂₉N₃O₃S₂ + H₂O: C, 57.8; H, 6.5; N, 8.8. Found: C, 58.1; H, 6.4; N, 8.3.

125 IR(neat): 3482, 3118, 2958, 1558, 1456 cm⁻¹

Example 13

The following compounds were also prepared in accordance with techniques described herein:

130 (i) *N*-butyloxycarbonyl-4-butyl-2-(4-imidazol-1-ylmethylphenyl)benzene-

sulfonamide

¹H NMR δ (CDCl₃): 8.95 (brs, 1H), 8.20 (d, J = 8.3 Hz, 1H), 7.66 (brs, 1H), 7.38 (d, J = 8.0 Hz, 2H), 7.34 (dd, J = 8.3, 1.6 Hz, 1H), 7.11 (d, J = 8.0 Hz, 2H), 7.05 (d, J = 1.6 Hz, 1H), 6.83 (brs, 2H), 5.10 (s, 2H), 4.00 (t, J = 6.5, 2H), 2.67 (t, J = 7.5 Hz, 2H), 1.62 (m, 2H), 1.48 (m, 2H), 1.36 (m, 2H), 1.22 (m, 2H), 0.92 (t, J = 7.2 Hz, 3H), 0.86 (t, J = 7.3 Hz, 3H)

¹³C NMR δ (CDCl₃): 151.9, 148.5, 140.2, 139.9, 136.7, 135.1, 134.7, 132.3, 130.8, 129.9, 127.7, 127.2, 126.9, 119.2, 65.9, 50.9, 35.4, 33.0, 30.5, 22.4, 18.8, 13.8, 13.6

- 10 (ii) *N*-(2-methoxyethyloxy)carbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide

¹H NMR δ (20% CD₃OD in CDCl₃): 8.00 (brs, 1H), 7.56 (d, J = 7.7 Hz, 2H), 7.19 (d, J = 7.7 Hz, 2H), 7.07 (brs, 2H), 6.75 (s, 1H), 5.20 (s, 2H), 4.14 (brt, J = 4.5 Hz, 2H), 3.52 (brt, J = 6.9 Hz, 2H), 3.32 (s, 3H), 2.70 (d, J = 7.1 Hz, 2H), 1.94 (m, 1H), 0.99 (d, J = 6.4 Hz, 6H)

15 ¹³C NMR δ (20% CD₃OD in CDCl₃): 153.1, 149.7, 144.0, 136.1, 134.8, 134.6, 132.8, 129.6, 128.8, 127.0, 125.6, 119.8, 70.0, 64.1, 58.2, 50.9, 38.9, 30.2, 21.8

- 20 (iii) *N*-ethyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide

¹H NMR δ (20% CD₃OD in CDCl₃): 7.78 (brs, 1H), 7.55 (d, J = 7.8 Hz, 2H), 7.24 (d, J = 7.8 Hz, 2H), 7.07 (brs, 2H), 6.77 (s, 1H), 5.22 (s, 2H), 4.06 (q, J = 7.1 Hz, 2H), 2.71 (d, J = 7.1 Hz, 2H), 1.95 (m, 1H), 1.17 (t, J = 7.1 Hz, 3H), 0.99 (d, J = 6.4 Hz, 6H)

25 ¹³C NMR δ (20% CD₃OD in CDCl₃): 153.0, 150.7, 145.1, 137.2, 135.9, 135.0, 132.7, 130.1, 129.5, 127.9, 127.6, 120.2, 62.6, 49.6, 39.5, 30.8, 22.3, 14.3

- (iv) *N*-*tert*-butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide

^1H NMR δ (10% CD_3OD in CDCl_3): 7.65 (brs, 1H), 7.39 (d, $J = 7.9$ Hz, 2H), 7.11 (d, $J = 7.9$ Hz, 2H), 6.94 (brs, 2H), 6.65 (s, 1H), 5.09 (s, 2H), 2.59 (d, $J = 7.1$ Hz, 2H), 1.82 (m, 1H), 1.22 (s, 9H), 0.87 (d, $J = 6.6$ Hz, 6H)

^{13}C NMR δ (10% CD_3OD in CDCl_3): 150.5, 149.5, 144.6, 136.6, 135.5, 134.2, 131.7, 129.5, 129.0, 128.0, 127.1, 119.6, 83.0, 50.6, 39.0, 30.3, 27.5, 21.9

(v) *N*-butyloxycarbonyl-3-[4-(4-methylimidazol-1-ylmethyl)phenyl]-5-*iso*-butylthiophene-2-sulfonamide

^1H NMR δ (CDCl_3): 8.22 (s, 1H), 7.55 (d, $J = 8.2$ Hz, 2H), 7.27 (d, $J = 7.9$ Hz, 2H), 6.84 (s, 1H), 6.74 (s, 1H), 5.18 (s, 2H), 4.03 (t, $J = 6.6$ Hz, 2H), 2.71 (d, $J = 6.9$ Hz, 2H), 2.24 (s, 3H), 1.94 (m, 1H), 1.51 (m, 2H), 1.28 (m, 2H), 1.00 (d, $J = 6.6$ Hz, 6H), 0.87 (t, $J = 7.4$ Hz, 3H)

^{13}C NMR δ (CDCl_3): 152.4, 150.5, 144.6, 135.2, 134.5, 132.6, 130.0, 129.1, 127.8, 117.1, 116.9, 66.3, 51.7, 39.3, 30.6, 22.3, 18.9, 13.6, 11.5

(vi) *N*-butyloxycarbonyl-3-(4-pyrazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide

^1H NMR δ (CDCl_3): 0.84 (t, $J = 7.3$ Hz, 3H), 0.95 (d, $J = 6.6$ Hz, 6H), 1.22 (m, 2H), 1.48 (m, 2H), 1.90 (m, 1H), 2.66 (d, $J = 6.9$ Hz, 2H), 4.01 (t, $J = 6.6$ Hz, 2H), 5.26 (s, 2H), 6.23 (s, 1H), 6.69 (s, 1H), 7.02 (d, $J = 8.3$ Hz, 2H), 7.37 (m, 3H), 7.48 (s, 1H)

^{13}C NMR δ (CDCl_3): 13.6, 18.7, 22.2, 30.4, 39.2, 55.1, 66.5, 106.0, 127.3, 129.3, 129.6, 133.7, 136.9, 139.4, 145.7, 150.6, 151.3

(vii) *N*-butyloxycarbonyl-3-[4-(3-trifluoromethylpyrazol-1-ylmethyl)phenyl]-5-*iso*-butylthiophene-2-sulfonamide

^1H NMR δ (CDCl_3): 7.66 (s, 1H), 7.50 (s, 1H), 7.42 (d, $J = 8.1$ Hz, 2H), 7.23 (d, $J = 8.2$ Hz, 2H), 6.74 (s, 1H), 6.59 (d, $J = 2.1$ Hz, 1H), 5.40 (s, 2H), 4.03 (t, $J = 6.6$ Hz, 2H), 2.71 (d, $J = 6.9$ Hz, 2H), 1.96 (m, 1H), 1.49 (m, 2H), 1.25 (m, 2H), 0.99 (d, $J = 6.6$ Hz, 6H), 0.87 (t, $J = 7.4$ Hz, 3H)

^{13}C NMR $\delta(\text{CDCl}_3)$: 151.8, 150.2, 146.0, 143.6, 142.5, 141.8, 139.4, 136.0, 134.0, 131.1, 130.6, 129.4, 127.5, 123.2, 119.2, 66.9, 56.1, 39.3, 30.5, 22.2, 18.7, 13.5

(viii) *N*-(*N*-butyl-*N*-methylamino)carbonyl-3-(4-imidazol-1-ylmethyl-

5 phenyl)-5-*iso*-butylthiophene-2-sulfonamide

^1H NMR $\delta(\text{CDCl}_3)$: 8.18 (brs, 1H), 7.84 (brs, 1H), 7.46 (d, $J = 8.2$ Hz, 2H), 7.16 (d, $J = 8.2$ Hz, 2H), 7.06 (brs, 1H), 6.99 (brs, 1H), 6.68 (s, 1H), 3.07 (brt, 2H), 2.67 (d, $J = 7.1$ Hz, 2H), 2.56 (brs, 3H), 1.91 (m, 1H), 1.35 (m, 2H), 1.19 (m, 2H), 0.96 (d, $J = 6.6$ Hz, 6H), 0.84 (t, $J = 7.2$ Hz, 3H)

10 ^{13}C NMR $\delta(\text{CDCl}_3)$: 153.6, 149.8, 143.3, 136.9, 135.5, 135.1, 134.2, 129.6, 128.7, 127.2, 126.8, 119.8, 51.0, 48.5, 39.3, 34.3, 30.4, 29.7, 22.3, 19.8, 13.8

(ix) *N*-butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-(2-methoxyethyl)thiophene-2-sulfonamide

15 ^1H NMR $\delta(5\% \text{CD}_3\text{OD in CDCl}_3)$: 7.86 (brs, 1H), 7.51 (d, $J = 7.9$ Hz, 2H), 7.18 (d, $J = 7.9$ Hz, 2H), 7.05 (brs, 1H), 6.99 (brs, 1H), 6.83 (s, 1H), 5.18 (s, 2H), 4.03 (t, $J = 6.5$ Hz, 2H), 3.67 (t, $J = 6.2$ Hz, 2H), 3.10 (t, $J = 6.2$ Hz, 2H), 1.52 (m, 2H), 1.26 (m, 2H), 0.88 (t, $J = 7.3$ Hz, 3H)

20 ^{13}C NMR $\delta(5\% \text{CD}_3\text{OD in CDCl}_3)$: 151.7, 148.1, 144.7, 136.9, 135.2, 134.8, 133.0, 129.9, 129.4, 127.4, 127.2, 119.8, 72.0, 66.3, 58.8, 51.1, 30.7, 30.5, 18.8, 13.6

Example 14

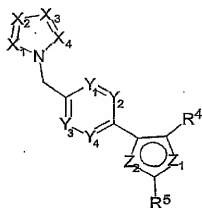
25 Title compounds of the Examples were tested in Tests A and B above and were found to exhibit an affinity for AT2 receptors of less than $K_i = 100$ nM (e.g. less than 50 nM) and an affinity to AT1 receptors of more than $K_i = 500$ nM (e.g. more than 1 μM).

Example 15

Title compounds of the Examples were tested in Test C above and were found to stimulate markedly mucosal alkalisation. This effect was blocked by co-administration of the selective AT₂ receptor antagonist PD123319
5 (Sigma Chemical Company).

Claims

1. A compound of formula I,



5

wherein

one of X_1 and X_2 represents $-N-$ and the other represents $-C(R^1)-$;

X_3 represents $-N-$ or $-C(R^2)-$;

10. X_4 represents $-N-$ or $-C(R^3)-$;

R^1 , R^2 and R^3 independently represent H, C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkoxy- C_{1-6} -alkyl or halo;

provided that, when X_1 represents $-C(R^1)-$, X_3 represents $-C(R^2)-$ and X_4 represents $-C(R^3)-$, then R^1 represents H;

15. Y_1 , Y_2 , Y_3 and Y_4 independently represent $-CH-$ or $-CF-$;

Z_1 represents $-CH-$, $-O-$, $-S-$, $-N-$ or $-CH=CH-$;

Z_2 represents $-CH-$, $-O-$, $-S-$ or $-N-$;

provided that:

- (a) Z_1 and Z_2 are not the same;
- 20 (b) when Z_1 represents $-CH=CH-$, then Z_2 may only represent $-CH-$ or $-N-$; and

(c) other than in the specific case in which Z_1 represents $-\text{CH}=\text{CH}-$, and Z_2 represents $-\text{CH}-$, when one Z_1 and Z_2 represents $-\text{CH}-$, then the other represents $-\text{O}-$ or $-\text{S}-$;

R^4 represents $-\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^6$, $-\text{S}(\text{O})_2\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^6$, $-\text{C}(\text{O})\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^6$,

5 or, when Z_1 represents $-\text{CH}=\text{CH}-$, R^4 may represent $-\text{N}(\text{H})\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^7$ or $-\text{N}(\text{H})\text{C}(\text{O})\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^7$;

R^5 represents C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkoxy- C_{1-6} -alkyl or di- C_{1-3} -alkylamino- C_{1-4} -alkyl;

R^6 represents C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkoxy- C_{1-6} -alkyl,

10 C_{1-3} alkoxy- C_{1-6} -alkoxy, C_{1-6} alkylamino or di- C_{1-6} alkylamino; and

R^7 represents C_{1-6} alkyl,

or a pharmaceutically-acceptable salt thereof.

2. A compound as claimed in Claim 1 wherein, when X_1 represents

15 $-\text{C}(\text{R}^1)-$, then X_3 represents $-\text{C}(\text{R}^2)-$ and X_4 represents $-\text{N}-$.

3. A compound as claimed in Claim 2 wherein R^1 represents H.

4. A compound as claimed in Claim 1 wherein, when X_1 represents

20 $-\text{C}(\text{R}^1)-$, then X_3 and X_4 both represent N.

5. A compound as claimed in Claim 1 wherein, when X_1 represents

$-\text{C}(\text{R}^1)-$, then X_3 represents $-\text{C}(\text{R}^2)-$ and X_4 represents $-\text{C}(\text{R}^3)-$.

25 6. A compound as claimed in Claim 1, wherein, when X_1 represents

$-\text{N}-$, then X_3 represents $-\text{N}-$.

7. A compound as claimed in Claim 6 wherein, when X_4 represents

$-\text{C}(\text{R}^3)-$, then R^3 represents H.

8. A compound as claimed in Claim 1, wherein, when X_1 represents $-N-$, then X_3 represents $-C(R^2)-$ and X_4 represents $-C(R^3)-$.
9. A compound as claimed in any one of Claims 1, 2, 4 or 6 to 8,
5 wherein R^1 represents H, C_{1-3} alkyl or CF_3 .
10. A compound as claimed in Claim 9, wherein R^1 represent H or ethyl.
11. A compound as claimed in any one of Claims 1 to 3, 5 or 8 to 10
10 wherein R^2 represents C_{1-3} alkyl, halo or H.
12. A compound as claimed in Claim 11 wherein R^2 represents H or methyl.
- 15 13. A compound as claimed in Claim 12 wherein R^2 represents H.
14. A compound as claimed in any one of Claims 1, 5, 6 or 8 to 13
wherein R^3 represents C_{1-3} alkyl, halo or H.
- 20 15. A compound as claimed in Claim 14 wherein R^3 represents H.
16. A compound as claimed in any one of the preceding claims wherein Y_1 , Y_2 , Y_3 and Y_4 all represent $-CH-$.
- 25 17. A compound as claimed in any one of the preceding claims wherein Z_1 represents $-S-$ or $-CH=CH-$.
18. A compound as claimed in Claim 17 wherein Z_1 represents $-S-$.

19. A compound as claimed in any one of the preceding claims wherein Z_2 represents $-\text{CH}-$.
20. A compound as claimed in any one of the preceding claims wherein R^4 represents $-\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^6$.
21. A compound as claimed in any one of the preceding claims wherein R^5 represents *n*-butyl or *iso*-butyl.
22. A compound as claimed in Claim 21 wherein R^5 represents *iso*-butyl.
23. A compound as claimed in any one of the preceding claims wherein, when R^4 represents $-\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^6$, $-\text{S}(\text{O})_2\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^6$ or $-\text{C}(\text{O})\text{N}(\text{H})\text{S}(\text{O})_2\text{R}^6$, R^6 represents *n*-butoxymethyl, *iso*-butoxy or *n*-butoxy.
24. A compound as claimed in Claim 23 wherein R^6 represents *n*-butoxy.
25. A compound as claimed in Claim 1 wherein, when X_1 , X_3 and X_4 all represent $-\text{CH}-$, Y_1 , Y_2 , Y_3 and Y_4 all represent $-\text{CH}-$, Z_1 represents $-\text{S}-$ or $-\text{CH}=\text{CH}-$, Z_2 represents $-\text{CH}-$ and R^5 represents *n*-butyl or *iso*-butyl, then R^4 represents $-\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^6$, in which R^6 represents $-\text{O}-n$ -butyl, $-\text{O}-iso$ -propyl, $-\text{O}-iso$ -butyl or $-\text{CH}_2-\text{O}-n$ -butyl.
26. *N*-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- N*-*iso*-butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- N*-*iso*-propyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;

- N*-(butoxyacetyl)-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- N*-butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-butylthiophene-2-sulfonamide;
- 5 *N*-butyloxycarbonyl-2-(4-imidazol-1-ylmethylphenyl)-4-*iso*-butylbenzene-sulfonamide;
- N*-butyloxycarbonyl-5-*iso*-butyl-3-(4-tetrazol-2-ylmethylphenyl)thiophene-2-sulfonamide;
- N*-butyloxycarbonyl-5-*iso*-butyl-3-(4-tetrazol-1-ylmethylphenyl)thiophene-
- 10 2-sulfonamide;
- N*-butyloxycarbonyl-3-(4-[1,2,4]triazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- N*-(butylamino)carbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- 15 *N*-butylsulfonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- N*-butylsulfonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-carboxamide;
- N*-butyloxycarbonyl-4-butyl-2-(4-imidazol-1-ylmethylphenyl)benzene-
- 20 sulfonamide;
- N*-(2-methoxyethyloxy)carbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- N*-ethyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- 25 *N*-*tert*-butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide;
- N*-butyloxycarbonyl-3-[4-(4-methylimidazol-1-ylmethyl)phenyl]-5-*iso*-butylthiophene-2-sulfonamide;
- N*-butyloxycarbonyl-3-(4-pyrazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-
- 30 2-sulfonamide;

- N*-butyloxycarbonyl-3-[4-(3-trifluoromethylpyrazol-1-ylmethyl)phenyl]-5-*iso*-butylthiophene-2-sulfonamide;
N-(*N*-butyl-*N*-methylamino)carbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-*iso*-butylthiophene-2-sulfonamide; or
- 5 *N*-butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)-5-(2-methoxyethyl)-thiophene-2-sulfonamide.
27. A pharmaceutical formulation including a compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, in
10 admixture with a pharmaceutically acceptable adjuvant, diluent or carrier.
28. A compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, for use as a pharmaceutical.
- 15 29. A compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, for use in the treatment of a condition in which selective agonism of the AT₂ receptor is desired and/or required.
- 20 30. A compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, for use in the treatment of a condition in which endogenous production of AngII is deficient.
31. A compound as defined in any one of Claims 1 to 26, or a
25 pharmaceutically acceptable salt thereof, for use in the treatment of a condition in which an increase in the effect of AngII is desired or required.
32. A compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, for use in the treatment of a

condition where AT2 receptors are expressed and their stimulation is desired or required.

33. The use of a compound as defined in any one of Claims 1 to 26, or a
5 pharmaceutically acceptable salt thereof, for the manufacture of a
medicament for the treatment of a condition in which selective agonism of
the AT2 receptor is desired and/or required.

34. The use of a compound as defined in any one of Claims 1 to 26, or a
10 pharmaceutically acceptable salt thereof, for the manufacture of a
medicament for the treatment of a condition in which endogenous
production of AngII is deficient.

35. The use of a compound as defined in any one of Claims 1 to 26, or a
15 pharmaceutically acceptable salt thereof, for the manufacture of a
medicament for the treatment of a condition in which an increase in the
effect of AngII is desired or required.

36. The use of a compound as defined in any one of Claims 1 to 26, or a
20 pharmaceutically acceptable salt thereof, for the manufacture of a
medicament for the treatment of a condition where AT2 receptors are
expressed and their stimulation is desired or required.

37. The use as claimed in any one of Claims 33 to 36, wherein the
25 condition is of the gastrointestinal tract, the cardiovascular system, the
respiratory tract, the kidneys, the eyes, the female reproductive (ovulation)
system, or the central nervous system.

38. The use as claimed in Claim 37, wherein the condition is oesophagitis,
30 Barrett's oesophagus, a gastric ulcer, a duodenal ulcer, dyspepsia (including

non-ulcer dyspepsia), gastro-oesophageal reflux, irritable bowel syndrome, inflammatory bowel disease, pancreatitis, hepatic disorders (including hepatitis), gall bladder disease, multiple organ failure, sepsis, xerostomia, gastritis, gastroparesis, hyperacidity, a disorder of the biliary tract, coelicia,

5 Crohn's disease, ulcerative colitis, diarrhoea, constipation, colic, dysphagia, vomiting, nausea, indigestion, Sjögren's syndrome, inflammatory disorders, asthma, an obstructive lung disease (including chronic obstructive lung disease), pneumonitis, pulmonary hypertension, adult respiratory distress syndrome, renal failure, nephritis, renal hypertension, diabetic retinopathy,

10 premature retinopathy, retinal microvascularisation, ovulatory dysfunction, hypertension, cardiac hypertrophy, cardiac failure, atherosclerosis, arterial thrombosis, venous thrombosis, endothelial dysfunction, endothelial lesions, post balloon dilatation stenosis, angiogenesis, diabetic complications, microvascular dysfunction, angina, cardiac arrhythmias, claudicatio

15 intermittens, preeclampsia, myocardial infarction, reinfarction, ischaemic lesions, erectile dysfunction, neointima proliferation, cognitive dysfunctions, dysfunctions of food intake (hunger/satiety), thirst, stroke, cerebral bleeding, cerebral embolus, cerebral infarction, hypertrophic disorders, prostate hyperplasia, autoimmune disorders, psoriasis, obesity,

20 neuronal regeneration, an ulcer, adipose tissue hyperplasia, stem cell differentiation and proliferation, cancer, apoptosis, tumours, hypertrophy diabetes, neuronal lesions or organ rejection.

39. The use as claimed in Claim 38, wherein the condition is non-ulcer

25 dyspepsia, irritable bowel syndrome, multiple organ failure, hypertension or cardiac failure.

40. A method of treatment of a condition in which selective agonism of the AT2 receptor is desired and/or required, which method comprises

30 administration of a therapeutically effective amount of a compound as

defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, to a person suffering from, or susceptible to, such a condition.

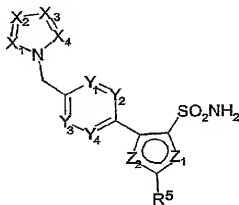
41. A pharmaceutical formulation including a compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, and an AT1 receptor antagonist, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier.
42. A kit of parts comprising components:
- (a) a pharmaceutical formulation including a compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier; and
- (b) a pharmaceutical formulation including an AT1 receptor antagonist, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier,
- which components (a) and (b) are each provided in a form that is suitable for administration in conjunction with the other.
43. A pharmaceutical formulation including a compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, and an angiotensin converting enzyme inhibitor, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier.
44. A kit of parts comprising components:
- (a) a pharmaceutical formulation including a compound as defined in any one of Claims 1 to 26, or a pharmaceutically acceptable salt thereof, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier; and

(b) a pharmaceutical formulation including an angiotensin converting enzyme inhibitor, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier,

which components (a) and (b) are each provided in a form that is suitable for administration in conjunction with the other.

45. A process for the preparation of a compound as defined in Claim 1, which comprises:

(i) for compounds of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ or $-S(O)_2N(H)S(O)_2R^6$, and R^6 is as defined in Claim 1, reaction of a compound of formula II,



II

wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as defined in Claim 1 with a compound of formula III,

15 R^6GL^1 III

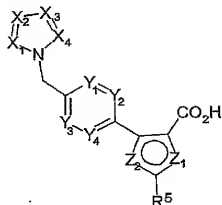
wherein G represents $C(O)$ or $S(O)_2$ (as appropriate), L^1 represents a suitable leaving group and R^6 is as defined in Claim 1;

(ii) for compounds of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ and R^6 represents C_{1-6} alkoxy- C_{1-6} -alkyl, coupling of a compound of formula II as defined above with a compound of formula IV,

20 $R^{6a}CO_2H$ IV

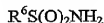
wherein R^{6a} represents C_{1-6} alkoxy- C_{1-6} -alkyl;

(iii) for compounds of formula I in which R^4 represents $-C(O)N(H)S(O)_2R^6$ and R^6 is as defined in Claim 1, coupling of a compound of formula V,



V

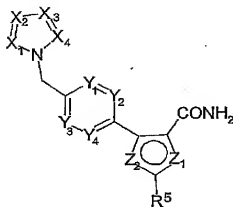
wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as defined in Claim 1, with a compound of formula VI,



VI

wherein R^6 is as defined in Claim 1;

(iv) for compounds of formula I in which R^4 represents $-C(O)N(H)S(O)_2R^6$ and R^6 is as defined in Claim 1, coupling of a compound of formula VII,



VII

10

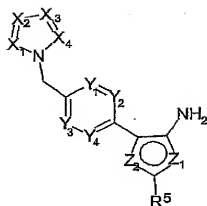
wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as defined in Claim 1, with a compound of formula VIII,



VIII

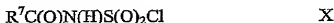
wherein R^6 is as defined in Claim 1;

15 (v) for compounds of formula I in which R^4 represents $-N(H)S(O)_2N(H)C(O)R^7$ and R^7 is as defined in Claim 1, reaction of a compound of formula IX,



IX

wherein X_1 , X_2 , X_3 , X_4 , Y_1 , Y_2 , Y_3 , Y_4 , Z_1 , Z_2 and R^5 are as defined in Claim 1, with a compound of formula X,



5 wherein R^7 is as defined in Claim 1;

(vi) for compounds of formula I in which R^4 represents $-N(H)C(O)N(H)S(O)_2R^7$ and R^7 is as defined in Claim 1, reaction of a compound of formula IX as defined above with a compound of formula XI,



10 wherein R^x represents C_{1-2} alkyl and R^7 is as defined in Claim 1;

(vii) for compounds of formula I in which R^4 represents $-N(H)C(O)N(H)S(O)_2R^7$ and R^7 is as defined in Claim 1, reaction of a compound of formula IX as defined above with a compound of formula XII,



15 wherein R^7 is as defined in Claim 1;

(viii) for compounds of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ and R^6 represents C_{1-6} alkylamino, reaction of a compound of formula II as defined above with a compound of formula XIII,



20 wherein R^{6b} represents C_{1-6} alkyl; or

(ix) for compounds of formula I in which R^4 represents $-S(O)_2N(H)C(O)R^6$ and R^6 represents di- C_{1-6} alkylamino, reaction of a corresponding compound of formula I in which R^4 represents

$-\text{S}(\text{O})_2\text{N}(\text{H})\text{C}(\text{O})\text{R}^6$ and R^6 represents C_{1-6} alkoxy with a compound formula XIV,



wherein R^{6c} and R^{6d} independently represent C_{1-6} alkyl.

5

46. A compound of formula II as defined in Claim 45 or a protected derivative thereof.

47. A compound of formula II as claimed in Claim 46, or a protected derivative thereof, wherein X_1 , X_3 and X_4 all represent $-\text{CH}-$, Y_1 , Y_2 , Y_3 and Y_4 all represent $-\text{CH}-$, Z_1 represents $-\text{S}-$ or $-\text{CH}=\text{CH}-$, Z_2 represents $-\text{CH}-$ and R^5 represents *n*-butyl or *iso*-butyl.

48. A compound of formula V as defined in Claim 45 or a protected derivative thereof.

49. A compound of formula VII as defined in Claim 45 or a protected derivative thereof.

50. A compound of formula IX as defined in Claim 45 or a protected derivative thereof.

20

INTERNATIONAL SEARCH REPORT

Int. and National Application No.

PCT/GB 02/02563

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07D233/54 A61K31/4174 A61P43/00 G01N33/00 C07D409/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07D A61K A61P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category * Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

A	US 5 444 067 A (SALAH KIVLIGHN ET AL.) 22 August 1995 (1995-08-22) cited in the application column 1 -column 2	1, 27, 33
A	WO 00 68226 A (AVENTIS PHARMA DEUTSCHLAND GMBH) 16 November 2000 (2000-11-16) cited in the application page 1; claim 1	1
A	EP 0 512 675 A (MERCK & CO., INC.) 11 November 1992 (1992-11-11) page 2; claims	1

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another claim or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

3 October 2002

Date of mailing of the international search report

11/10/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2260 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Van Bijlen, H

INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/GB 02/02563

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
US 5444067	A	22-08-1995	GB	2281298 A	01-03-1995
WO 0068226	A	16-11-2000	DE	19920815 A1	09-11-2000
			DE	19961686 A1	28-06-2001
			AU	4753600 A	21-11-2000
			BR	0010248 A	13-02-2002
			CN	1349530 T	15-05-2002
			CZ	20013907 A3	13-02-2002
			WO	0068226 A1	16-11-2000
			EP	1185527 A1	13-03-2002
			NO	20015309 A	28-12-2001
			TR	200103171 T2	21-06-2002
			US	2002077344 A1	20-06-2002
			US	6235766 B1	22-05-2001
			US	2001018449 A1	30-08-2001
EP 512675	A	11-11-1992	US	5198438 A	30-03-1993
			CA	2063895 A1	08-11-1992
			EP	0512675 A1	11-11-1992
			JP	5140153 A	08-06-1993

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT D

Surface epithelial HCO_3^- transport by mammalian duodenum in vivo

G. FLEMSTRÖM, A. GARNER, O. NYLANDER, B. C. HURST, AND J. R. HEYLINGS
Department of Physiology and Medical Biophysics, Uppsala University Biomedical Center,
S-751 23 Uppsala, Sweden; and Bioscience Department, Imperial Chemical Industries
Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire SK10 4TG, United Kingdom

FLEMSTRÖM, G., A. GARNER, O. NYLANDER, B. C. HURST, AND J. R. HEYLINGS. Surface epithelial HCO_3^- transport by mammalian duodenum in vivo. *Am. J. Physiol.* 243 (Gastrointest. Liver Physiol. 6): G348-G358, 1982.—Duodenal surface epithelial transport of HCO_3^- was measured by direct titration in anesthetized animals. Alkalinization of the lumen occurred in all species, although basal rates varied considerably: rats (~10), cats (~15), pigs (~25), dogs (~25), guinea pigs (~40), and rabbits (~170 $\mu\text{eq}\cdot\text{cm}^{-1}\cdot\text{h}^{-1}$). In cats duodenum transported HCO_3^- at a greater basal rate than jejunum (~5 $\mu\text{eq}\cdot\text{cm}^{-1}\cdot\text{h}^{-1}$) and developed a higher transmucosal electrical potential difference (PD, lumen negative). Luminal application of 10 mM HCl for 5 min produced a sustained increase in the rate of duodenal HCO_3^- transport that was accompanied by a rise in appearance of E-like prostaglandin immunoreactivity in the lumen and a decrease in DNA release. In cats pretreated with indomethacin (10 mg/kg iv), acid caused only a transient increase in HCO_3^- transport. Exogenous prostaglandin E_2 (1–12 μM , luminal) increased basal HCO_3^- transport in cats, rats, and dogs but had no effect on this transport in guinea pigs and rabbits. However, prostaglandin E_2 increased HCO_3^- transport and PD in guinea pigs pretreated with inhibitors of tissue cyclooxygenase activity (indomethacin or aspirin) or gastric H^+ secretion (cimetidine). Thus the continuous exposure of the duodenum of herbivores to HCl discharged from the stomach may itself stimulate HCO_3^- transport via an increase in endogenous prostaglandin levels and render exogenous prostaglandins ineffective. Secretin (1–15 CU/kg iv) was without effect in both cats and guinea pigs. In guinea pigs, intravenous glucagon (120–360 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) or gastric inhibitory peptide (5 $\mu\text{g}/\text{kg}$) both increased HCO_3^- transport but not PD. Hence, prostaglandin-stimulated and hormone-stimulated mechanisms of HCO_3^- transport probably occur in mammalian duodenum as found previously in the isolated amphibian duodenum. The results suggest that epithelial HCO_3^- transport is a major mechanism of acid disposal, and thus mucosal protection, in mammalian duodenum under the control of hormones and endogenous prostaglandins.

acid disposal; gut hormones; mucosal protection; prostaglandins

MECHANISMS by which gastric and duodenal mucosa are normally able to resist intraluminal acid are not fully understood. In vitro and in vivo studies on the properties of gastric HCO_3^- transport have highlighted the potential role of a surface "mannos-bicarbonate" barrier in H^+ disposal and mucosal protection (5). Most of the acid secreted by the stomach passes into the duodenum where neutrality is restored. Although pancreatic and biliary

HCO_3^- is important in terms of bulk neutralization, it is clear that duodenal mucosa itself has an independent mechanism for H^+ disposal. Florey and collaborators (7) originally showed that the ability of duodenum to resist intraluminal acid was considerably greater than in lower parts of the small intestine and was unrelated to secretion of pancreatic or biliary HCO_3^- . It was proposed that duodenal resistance to acid was due to neutralization by an alkaline fluid originating from Brunner's glands or of other mucosal origin. Ulcers are infrequent in patients with occulting carcinoma of the papilla of Vater (28), which seems to preclude an obligatory role for pancreatic HCO_3^- in duodenal mucosal protection in humans. Subsequent experiments have confirmed the capacity of duodenal epithelium to dissipate acid loads (10, 14, 33). Proposed mechanisms include ultrafiltration and/or secretion of HCO_3^- , H^+ backdiffusion, or a combination of these. Furthermore, duodenal secretion of HCO_3^- may be independent of Brunner's glands because, in dogs (10) and humans (33), proximal and distal segments of duodenum display a similar ability to remove acid, although these glands are mainly confined to the proximal region.

The mechanism and properties of surface epithelial HCO_3^- transport by proximal duodenum devoid of Brunner's glands have recently been studied in vitro. An isolated bullfrog preparation transported HCO_3^- at a steady basal rate (~1.0 $\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$) and developed a potential difference (PD) of 3–10 mV, lumen negative (3, 6, 29). Rates of HCO_3^- transport into the lumen in duodenum were considerably higher than either stomach or jejunum, and both the mechanism of transport and stimulatory and inhibitory pathways differ substantially from those of gastric mucosa (5). Thus, gastric transport in vitro occurs by (electroneutral) $\text{HCO}_3^-/\text{Cl}^-$ exchange at the luminal membranes of surface epithelial cells. In duodenum, however, in addition to $\text{HCO}_3^-/\text{Cl}^-$ exchange stimulated by hormones and sensitive to furosemide together with passive transmucosal migration of some HCO_3^- , there is probably electrogenic transport of this ion. The latter component is independent of luminal Cl^- and stimulated by dibutyl cAMP and prostaglandins. As in the stomach, duodenal HCO_3^- transport is sensitive to inhibitors of tissue metabolism.

The aim of the present study was to develop a method to enable direct measurement of duodenal surface epithelial HCO_3^- transport in mammals in vivo and to com-

pare properties with those of the amphibian duodenum in vitro. In situ segments of duodenum, devoid of Brunner's glands and free from bile and pancreatic secretions, were investigated. Experiments were performed on anesthetized cats and guinea pigs as well as on other mammals. Some of the results have been presented in a preliminary form at a symposium (4).

METHODS

Animals. The following were used: rats (300–400 g), guinea pigs (400–700 g), cats (2–3 kg), rabbits (3–4 kg), dogs (10–15 kg), and pigs (30–35 kg). All were males with the exception of cats and dogs, which were of either sex. Animals were obtained from Imperial Chemical Industries Pharmaceuticals Division animal breeding unit with the exception of guinea pigs (Sahlin, Malmö, Sweden) and Sprague-Dawley rats (Anticimex, Stockholm, Sweden). Animals were starved for 20–24 h before use and allowed free access to drinking water. Rodents were starved in cages with wide-mesh bottoms to prevent coprophagy.

Guinea pig preparation. Guinea pigs were anesthetized with 1.3 g/kg urethane intraperitoneally, and body temperature was maintained at 37°C by means of a heated blanket controlled by an intrarectal thermistor. The trachea was cannulated and the abdominal cavity was opened by a midline incision. Excess urethane within the peritoneal cavity was removed by irrigation with 0.15 M NaCl at 37°C . A 12-mm segment of the descending part of the duodenum starting 5 mm distal to the outlet of the bile duct, and thus proximal to the pancreatic duct, was cannulated in situ between the two glass limbs of a reservoir (Fig. 1). To prevent exposure of duodenum proximal to the segment under study to bile or gastric juice, the bile duct and pylorus were ligated (see Fig. 2) before closing the abdomen with sutures. To prevent dehydration, isotonic NaCl ($1 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) was infused

intravenously throughout the experiment. The chamber contained 4 ml of 0.15 M NaCl, which was maintained at 37°C with a water jacket and was circulated rapidly through the duodenal segment by a gas lift of 100% O_2 prewashed in $\text{Ba}(\text{OH})_2$. Alkalinization of the luminal fluid was titrated at end points of pH 7.4 or 8.0 by infusion of 25 or 50 mM HCl made isotonic with NaCl. Titrant delivery was controlled automatically by a pH electrode (GK 2321C, Radiometer, Copenhagen, Denmark) connected to pH-stat equipment (ABU 80, PHM 62, TTT 80, Radiometer). Transmucosal electrical PD was recorded on a high-input-impedance voltmeter via matched calomel half-cells. The half-cells were connected by means of agar bridges (2 M KCl) with their distal ends located in the luminal solution and superior vena cava. Asymmetry between the two ends of the measuring system ($<1 \text{ mV}$) was determined by placing distal ends of the two agar bridges in a solution of 2 M KCl. To avoid induction of H^+ diffusion potentials in the bridge, PD was not recorded when acidic luminal solutions were used.

Cat preparation. Anesthesia was induced by halothane (Fluothane, Imperial Chemical Industries) and maintained with chloralose (100 mg/kg iv). A 16-mm segment of duodenum, starting just distal to the papilla of Vater, was cannulated for perfusion, and the common bile duct and pylorus were ligated (Fig. 2). A larger chamber and greater volume of perfusate (20 ml of 0.15 M NaCl) were used in the cat. The duodenal segment under study was always checked for accessory pancreatic ducts, which may occur in this species (22), and they were ligated if present. The rate of alkalinization of the luminal circulating solution and PD between the duodenal lumen and inferior vena cava were measured as described for guinea pigs. In some experiments, a 16-mm segment of jejunum, starting 15 cm distal to the pylorus, was also cannulated, and its properties were studied simultaneously with the duodenum.

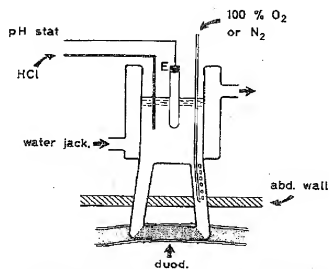


FIG. 1. Diagram of glass chamber used to perfuse segments of duodenum. Fluid (0.15 M NaCl), maintained at 37°C by means of a water jacket, was circulated by a gas lift (100% O_2 or N_2). Rate of alkaline secretion was determined by infusion of HCl controlled by a pH-stat system.

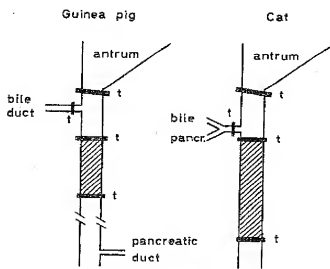


FIG. 2. Location of duodenal segments under study in guinea pig and cat. In guinea pig, chamber was positioned distal to bile duct and proximal to pancreatic duct, whereas in cat perfused segment was distal to common bile duct (t indicates ligatures). These segments were devoid of Brunner's glands.

Other species. Apart from the anesthetic, chamber dimensions, and volume of circulating fluid, experimental techniques were similar to those described for guinea pigs and cats. Rats were anesthetized with 120 mg/kg ip inactin (Promonta, Hamburg, W. Germany) and rabbits with 1.3 g/kg iv urethane after halothane induction. Anesthesia was induced with 25 mg/kg iv thiopental sodium (Intraval, May & Baker, Dagenham, UK) in dogs and 1.0 ml/kg im Imobilon (Reckitt & Colman, Hull, UK) in pigs. Anesthesia was maintained by halothane in dogs and pigs. The titration chamber for rats (12-mm segment perfused with 4 ml) was the same as for guinea pigs, and for rabbits (16-mm segment perfused with 20 ml) it was the same as for cats. In dogs and pigs, a 30-mm segment of duodenum was perfused with 50 ml of 0.15 M NaCl. The location of duodenal segments examined with respect to bile and pancreatic ducts (see Ref. 22) was as follows: rats, distal to outlet of common bile duct (~2 cm from pylorus); rabbits, distal to pancreatic (and bile) ducts (~5 cm from pylorus); dogs, distal to larger pancreatic (and common bile) ducts (~8 cm from pylorus); and pigs, distal to common bile and proximal to the pancreatic duct (~4 cm from pylorus).

Experimental protocol and drugs. After completion of surgery, luminal alkalization and PD were allowed to stabilize for 45–60 min. Rates of alkalization were determined from the amount of acid titrant infused over 10-min intervals and expressed as microequivalents of base per centimeter of duodenum per hour. For intravenous administration of drugs or NaCl, the left femoral vein was also cannulated. In cats and rats the influence of topical acid was determined by replacing the circulating fluid with a solution of 1 or 10 mM HCl made isotonic with NaCl. After 5 min, the acid was removed and the reservoir was rinsed and refilled with 0.15 M NaCl. During acid instillation experiments in rats, mean arterial blood pressure (right femoral artery) was recorded using a conventional mercury manometer. In some experiments, guinea pigs were pretreated with the histamine H_2 -receptor antagonist cimetidine (Smith Kline & French, Welwyn Garden City, UK) to reduce gastric acid secretion. The drug was given in a dose of 20 mg/kg sc twice daily for the 3 days preceding an experiment and 20 mg/kg 2 h before surgery.

Prostaglandins E_2 (PGE $_2$, Sigma Chemical, St. Louis, MO) and 16,16-dimethyl E_2 (Imperial Chemical Industries) were dissolved in ethanol, stored at -20°C, and added to the luminal solution (topical administration) in a small volume (<30 μ l) from a concentrated stock solution (10⁻⁴ M). Indomethacin and aspirin were purchased from Sigma Chemical. Stock solutions of indomethacin were prepared on the day of use by dissolving in ethanol and phosphate buffer (pH 7.4). Glucagon (pancreatic, mixture of porcine and bovine) was purchased from Sigma Chemical; gastric inhibitory peptide (GIP, porcine) from Peninsula Laboratories, San Carlos, CA; and secretin (porcine) from Kabi Diagnostics, Stockholm, Sweden. Glucagon was dissolved at pH 8.9 and then pH was adjusted to 7.4 with HCl. GIP and secretin were dissolved in isotonic sterile saline at pH 7.2. Stock solutions of all hormones were prepared on the day of use and contained (0.5 mg/ml) bovine serum albumin (Sigma Chemical, crystallized). The hormones, indomethacin,

and aspirin were administered by slow intravenous injection in a volume not exceeding 1 ml/kg. Luminal administration of cyclooxygenase inhibitors was avoided because high luminal concentrations of aspirin induce mucosal damage and increase permeability to ions, including HCO_3^- (5).

The statistical significance of data presented in this paper was determined using Student's *t* test for paired variates, comparing results during exposure to drugs with those in preceding control periods.

Morphological examinations. In the case of guinea pigs and cats, the duodenal segment studied was saved from every third or fourth animal for histology. Two specimens from each of the other species were also examined. Tissues were fixed in formal-saline (10%), embedded in paraffin wax, and sectioned longitudinally (7 μ m thick). Sections were stained with hematoxylin and eosin and observed by light microscopy for the presence of Brunner's glands and any morphological changes. There were no Brunner's glands in any of the sections from any of the species used with the exception of pigs in which a large number was present. Duodenal segments from four cats examined after exposure to HCl (1–10 mM) and PGE $_2$ (6 μ M) showed a normal histological appearance and were indistinguishable from control (untreated) specimens.

Estimation of DNA and E-like prostaglandin (PGE). Concentrations of DNA and PGE in cat duodenal perfusates were measured by radioimmunoassay (16, 20). Samples (1 ml) of perfusate were taken every 10 min during acid instillation experiments and stored at -20°C. Circulating volume was kept constant at 20 ml by addition of 0.15 M NaCl.

For determination of DNA, aliquots of perfusate and 0.1 M Tris buffer (50 μ l of each) were incubated with 0.08 μ Ci of tracer obtained from anti-DNA kit TM 76 (The Radiochemical Centre, Amersham, UK) and human systemic lupus erythematosus-positive serum, diluted to bind approximately 50% of the tracer. Residual tracer was separated by precipitation with saturated $(NH_4)_2SO_4$ and centrifugation (1,500 g for 15 min) at 4°C, and activity was determined in an automatic gamma counter (LKB Instruments, Turku, Finland). The amount of DNA was calculated by comparison with standard displacement curves of calf thymus DNA (Sigma Chemical) in the range 0.05–5.0 μ g. The antiserum cross-reacted by only 0.4% with RNA and not at all with either sialic acid or heparin.

PGE-like immunoreactivity in 100- μ l aliquots from the same duodenal samples was measured by comparison with the displacement of [3H]PGE $_2$ (TRK 431, The Radiochemical Centre) from anti-PGE antiserum (PRE 4, Miles Yeda, Slough, UK) by standard amounts of PGE $_2$ in the range 15–200 pg. Tracer remaining in 0.1-ml aliquots of supernatant obtained by centrifugation (2,000 g for 15 min) at 4°C, after adsorption of unbound prostaglandin on dextran-coated charcoal, was estimated by liquid scintillation spectrometry (Phillips, Rotterdam, Holland).

RESULTS

Evaluation of technique. Duodenum in all species examined (rats, guinea pigs, cats, rabbits, dogs, and pigs)

alkalinized the luminal solution as determined by pH-stat titration at an end point of pH 7.4. Apart from rabbits and pigs, in which no measurements were taken, the duodenum of each species developed a transepithelial electrical PD (1–10 mV, lumen negative). In cats, rats, and guinea pigs, there was a decrease in luminal alkalization in animals in which the level of anesthesia was either too superficial or too deep. Special attention was therefore paid to maintain anesthesia at stage 3a, and experiments in which this level could not be satisfactorily controlled have been excluded. Similarly, stretching the mesentery during operation increased alkalization transiently (~30 min) in both cats and guinea pigs, while intravenous bolus injection of larger (>2.0 ml in cats and >0.5 ml in guinea pigs) volumes also resulted in transient increases in duodenal alkalization. Care was thus taken to ensure rigid positioning of titration chamber, animal, and associated apparatus throughout experiments. Drugs and hormones were only administered when steady basal rates of transport had been recorded for 45 min or longer and the volume of fluid injected intravenously was kept to the minimum. Careful control of body temperature and a constant rate of gassing of the luminal perfusate were also necessary to obtain stable rates of alkalization.

During surgery, extreme care was taken to avoid tying off blood vessels supplying the segment under study or adjacent tissue. These were checked for normal color and configuration after completion of surgical procedures and at the end of experiments. Change of gassing from 100% O₂ to 100% N₂ for a 30-min period in cats or guinea pigs ($n = 6$ for both) had no effect on either the rate of duodenal luminal alkalization or PD, further indicating that vascular perfusion of the tissue segments was adequate. Interference with blood supply by tying off vessels supplying segments of cat duodenum or jejunum ($n = 4$ for both) resulted in net acidification of the luminal solution of both tissues and was accompanied by discoloration of the mucosa. These observations may be compatible with the luminal acidification by duodenal and jejunal mucosa observed in some mammalian preparations *in vitro* (21).

To preclude the possibility that passive diffusion of H⁺ from the luminal solution contributed to measured rates of alkalization, luminal pH was always maintained above tissue pH. A titration end point of pH 7.4 was used routinely. However, pH 8.0 was used in some experiments with the guinea pig ($n = 8$) and gave values for transport rate that were identical to those measured at end-point pH 7.4. Titration thus necessitated efficient removal of CO₂ formed during reaction between titrant (HCl) and secreted HCO₃⁻ together with any CO₂ that may diffuse into the luminal solution from the tissue (5). With the design of chambers and (rapid) gassing by O₂ used in the present study, 93–102% of exogenous HCO₃⁻ (5–20 μ eq) was titrated within 5 min of addition ($n = 12$). Exogenous NaOH was titrated more rapidly (within 2 min). This difference probably reflects time required for conversion of some HCO₃⁻ to CO₂^{*} and removal of CO₂.

Basal HCO₃⁻ transport and PD. Basal rates of duodenal epithelial HCO₃⁻ transport and transmucosal PD (lumen negative) are shown in Table 1. Although alkalization of the luminal solution was observed in all six

species, transport rates were not directly comparable due to differences in diameter (and thus in surface area) of tissue between the limbs of the perfusion chamber. Nevertheless, HCO₃⁻ transport by rabbit duodenum was considerably greater than in other species studied. Pigs were the only species in which Brunner's glands were present in the segment under study. This was not associated with higher HCO₃⁻ rates.

In the cat, basal rates of HCO₃⁻ transport and PD in duodenum and jejunum were compared in the same animal (Table 2). Both were considerably greater in the duodenum.

Stimulation by luminal acid. Effects of luminal (topical) application of 1 and 10 mM HCl (NaCl to isotonicity) on HCO₃⁻ transport in the cat are shown in Fig. 3. A slight but nonsignificant ($P > 0.05$) rise in alkalization occurred after instillation of 1 mM HCl for 5 min, while exposure to 10 mM HCl significantly ($P < 0.01$) increased HCO₃⁻ transport. After exposure to 10 mM HCl the rate of duodenal HCO₃⁻ transport remained elevated within the experimental periods tested in the present study (up to 80 min). Administration of indomethacin (10 mg/kg iv) inhibited ($0.01 < P < 0.02$) this sustained response to acid. There were no significant changes in PD associated with stimulation by luminal acid or inhibition of transport by indomethacin. The effect of luminal HCl was also determined in cats given indomethacin before exposing the duodenal lumen to acid (Fig. 4). Basal rates were lower in these indomethacin-treated animals as compared with the control group (2.5 and 5.4 μ eq·cm⁻¹·h⁻¹). Further, exposure of the duodenum to 10 mM luminal HCl for 5 min caused a marked increase in the

TABLE 1. Duodenal HCO₃⁻ transport and PD in different mammals

Species	Basal Values			PGE ₂ , 6 μ M	
	HCO ₃ ⁻ , μ eq·cm ⁻¹ ·h ⁻¹	PD, mV	n	HCO ₃ ⁻ , % increase	n
Rat	10.0 \pm 0.8	0.9 \pm 0.1	44	84	7
Cat	16.9 \pm 2.2	2.9 \pm 0.2	82	138	16
Pig	24.6 \pm 5.7	Not measured	3	Not tested	
Guinea pig	25.9 \pm 13.4	3.9 \pm 1.5	6	103	4
Dog	29.0 \pm 3.1	1.6 \pm 0.6	35	No effect	9
Rabbit	154.4 \pm 21.1	Not measured	8	No effect	3

Values are means \pm SE; n, no. of animals. Steady-state control values for the rate of duodenal HCO₃⁻ transport and transepithelial electrical potential difference (PD, lumen negative) are shown. The mean percentage increase in rate of HCO₃⁻ transport 30 min after luminal (topical) administration of 6 μ M prostaglandin E₂ (PGE₂) is also given.

TABLE 2. Comparison of duodenal and jejunal HCO₃⁻ transport and PD in the cat

Tissue	Basal Values		PGE ₂ , 6 μ M	
	HCO ₃ ⁻ , μ eq·cm ⁻¹ ·h ⁻¹	PD, mV	HCO ₃ ⁻ , μ eq·cm ⁻¹ ·h ⁻¹	PD, mV
Duodenum	16.9 \pm 3.3	2.8 \pm 0.6	37.8 \pm 6.2	4.5 \pm 1.0
Jejunum	5.4 \pm 1.4	0 \pm 0.5	9.2 \pm 2.3	1.1 \pm 0.6

Means \pm SE of steady-state values are shown for duodenum and jejunum from the same cat ($n = 8$). Prostaglandin E₂ (PGE₂) was applied luminally, and the rate of HCO₃⁻ transport and transepithelial electrical potential difference (PD, lumen negative) recorded 30 min after addition are given.

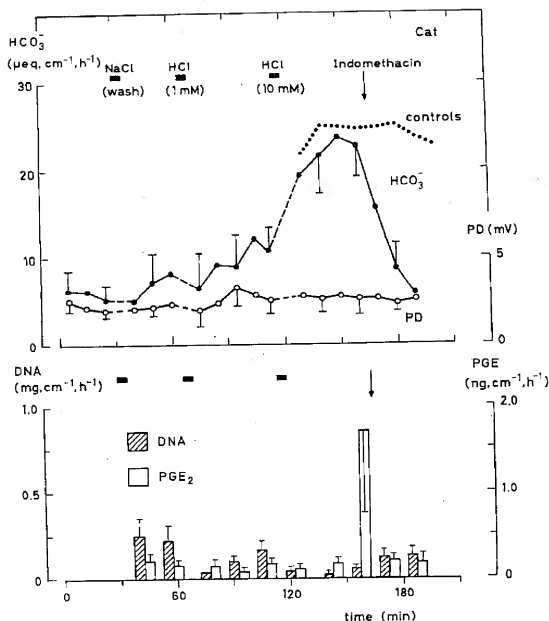


FIG. 3. Influence of luminal acidification (1 and 10 mM HCl for 5 min) on rate of HCO_3^- transport and transepithelial electrical potential difference (PD) in cat duodenum. *Upper panel:* outputs of DNA and prostaglandin E-like immunoreactivity into circulating solutions. Acid (10 mM HCl) stimulated HCO_3^- transport, and this response was inhibited by indomethacin (10 mg/kg iv, $n = 5$). There was no decline in acid-treated controls not given indomethacin (dotted line, $n = 3$). Increase in HCO_3^- transport was accompanied by a rise in prostaglandin release and a fall in DNA output. Mean values \pm SE are shown.

rate of alkalization, but the response was transient in comparison with that recorded in animals not pretreated with indomethacin.

The influence of luminal acidification on levels of DNA and prostaglandin E-like activity in the circulating fluid were used as indices of mucosal cell loss and endogenous prostaglandin production (Fig. 3). A decrease in DNA output occurred after exposure to both 1 and 10 mM HCl. Acid itself (up to 100 mM HCl) did not interfere with the radioimmunoassay of DNA. The concentration of PGE-like immunoreactivity in duodenal perfusates was not altered after exposure to 1 mM HCl, but a sharp increase in mean output ($0.02 < P < 0.05$) occurred 35–45 min after 10 mM HCl. There was an immediate return to preexposure levels after administration of indomethacin.

Effects of luminal HCl were also tested in rats (Fig. 5) and guinea pigs. In the former, topical acid induced a small but sustained increase in HCO_3^- transport ($P < 0.01$) that was inhibited ($P < 0.01$) by aspirin (30 mg/kg

iv). The increase in duodenal HCO_3^- transport in the rat in response to acid was thus similar in profile but smaller in magnitude than in the cat. In addition, in rats there were no changes in mean arterial blood pressure accompanying either stimulation or inhibition of HCO_3^- transport. Basal rates of transport in segments of guinea pig duodenum were higher than in cats or rats, and in guinea pigs 10 mM HCl had no significant ($P > 0.05$, $n = 4$) effect on duodenal HCO_3^- transport.

Effects of exogenous prostaglandins and cyclooxygenase inhibitors. In the basal secreting cat, PGE₂ caused a dose-dependent rise in HCO_3^- transport ($P < 0.01$ at 6 μM) and PD ($0.01 < P < 0.02$ at 6 μM) when added to the luminal solution (Fig. 6). Exogenous PGE₂ (6 μM , $n = 7$) also reversed ($P < 0.01$) the inhibitory action of indomethacin on acid-stimulated HCO_3^- transport (cf. Fig. 3), and this response was accompanied by a rise in PD. In the rat, 3–12 μM PGE₂ (luminal) induced a dose-dependent stimulation of duodenal alkalization ($P < 0.01$ at 12 μM , $n = 7$), but there was no significant

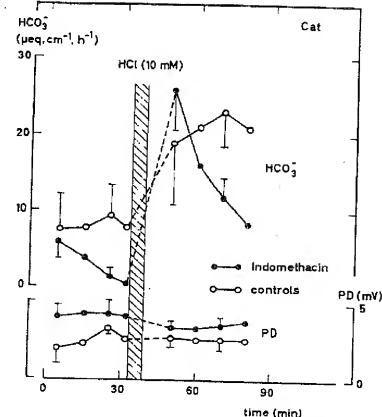


FIG. 4. Effect of pretreatment with indomethacin (10 mg/kg iv) on HCO_3^- transport in response to luminal acidification (10 mM HCl for 5 min) in cat duodenum. Compared with untreated animals, in which increase in HCO_3^- transport was sustained, cats pretreated with indomethacin showed only a transient response to acid. Mean values \pm SE are shown ($n = 5$).

increase in PD ($P > 0.05$) in this species. A single concentration only (6 μM , $n = 4$) was used in the dog, and duodenal epithelial HCO_3^- transport rose from 27.5 ± 6.6 to $55.8 \pm 16.7 \mu\text{eq}\cdot\text{cm}^{-1}\cdot\text{h}^{-1}$ ($0.02 < P < 0.05$) 30 min after administration. Effects of prostaglandin were not tested in the pig. As in the duodenum, jejunal transport of HCO_3^- and transmucosal PD in the cat were increased by luminal PGE_2 (Table 2). The magnitude of these increases were similar such that the ratio between duodenal and jejunal transport rates and PD (duodenum greater) remained unchanged.

In contrast to the findings in cats, rats, and dogs, PGE_2 (6 μM) had no effect on basal duodenal HCO_3^- transport or PD in either guinea pigs or rabbits (Table 1). Likewise, the synthetic analogue 16,16-dimethyl PGE_2 (2–10 μM , $n = 4$) was without effect on basal transport (or PD) in the guinea pig.

A species difference was also observed with respect to the action of inhibitors of tissue prostaglandin synthesis. Thus, indomethacin (10 mg/kg iv, $n = 6$), although causing a slight decrease, had no significant ($P > 0.05$) effect on basal HCO_3^- transport or PD in the cat. In the guinea pig, indomethacin (10 or 25 mg/kg) inhibited basal transport ($0.01 < P < 0.02$ for both doses) and also decreased PD (Fig. 7). Similarly, aspirin (10–30 mg/kg, $n = 5$) decreased basal HCO_3^- transport ($P < 0.01$) in the guinea pig (Fig. 8) but had no effect in the cat ($n = 2$).

Inhibition of basal HCO_3^- transport in guinea pigs but

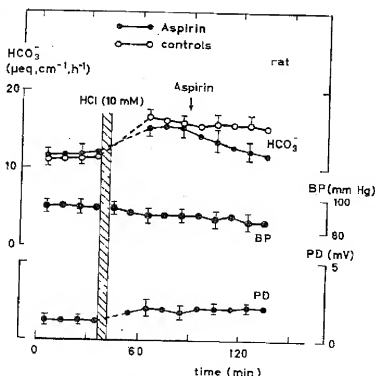


FIG. 5. Luminal acidification with 10 mM HCl for 5 min stimulated duodenal HCO_3^- transport in rat. This response was sensitive to aspirin (30 mg/kg iv). There was a slight increase in transmembrane potential difference (PD) in response to acid, but neither increase nor decrease in HCO_3^- transport was associated with changes in mean arterial blood pressure (BP). Mean values \pm SE are shown in controls exposed to acid only ($n = 5$) and after administration of aspirin ($n = 8$).

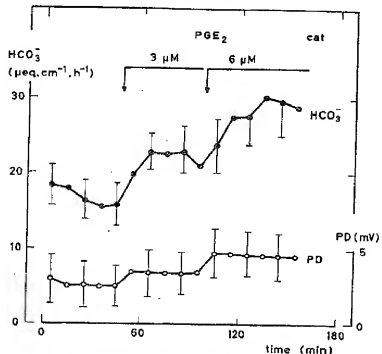


FIG. 6. Luminal administration of prostaglandin E_2 (PGE_2 , 3–6 μM) produced a dose-dependent increase in duodenal HCO_3^- transport and transmembrane potential difference (PD) in cat. Mean values \pm SE are shown ($n = 5$).

not cats by cyclooxygenase inhibitors suggested that, in the former species, high levels of endogenous prostaglandin might render duodenal epithelium insensitive to ex-

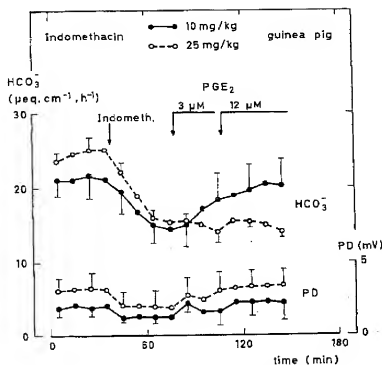


FIG. 7. Effects of indomethacin (10 and 25 mg/kg iv) on HCO_3^- transport in guinea pig duodenum. Both doses inhibited HCO_3^- transport and reduced potential difference (PD). At lower dose of indomethacin (10 mg/kg) secretion recovered in response to luminal prostaglandin E_2 (PGE_2), whereas at higher dose (25 mg/kg) secretion was insensitive to subsequent administration of this prostaglandin. Mean values \pm SE are shown ($n = 8$ for both series).

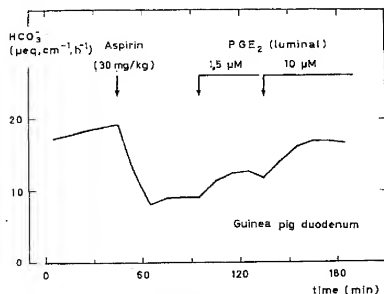


FIG. 8. Experiment to illustrate effect of aspirin and prostaglandin E_2 (PGE_2) on duodenal HCO_3^- transport in guinea pig. Although PGE_2 (up to 10 μM luminally) had no effect in basal secreting preparations, this agent caused a dose-dependent stimulation of duodenal HCO_3^- transport in animals pretreated with aspirin (30 mg/kg iv).

ogenous administration of these agents. Effects of exogenous PGE_2 were therefore tested in guinea pigs pretreated with intravenous indomethacin (Fig. 7) or aspirin (Fig. 8). In both cases, HCO_3^- transport was stimulated by luminal PGE_2 ($P < 0.01$ in animals treated with 10 mg/kg indomethacin or 30 mg/kg aspirin). However,

exogenous PGE_2 had no effect in guinea pigs treated with a high dose (25 mg/kg) of indomethacin.

Being exclusively herbivorous animals, the duodenum of guinea pigs (and rabbits) should be continuously exposed to acid discharged from the stomach. The influence of exogenous PGE_2 was therefore also determined in guinea pigs pretreated with the histamine H_2 -receptor antagonist cimetidine to reduce gastric H^+ secretion. In these animals, PGE_2 (12 μM) increased HCO_3^- transport (~30%) and PD (~40%) in four out of six experiments. The cat is an intermittent feeder and, although PGE_2 (6 μM) stimulated basal HCO_3^- transport in untreated cats (Fig. 6), it had no further effect in cat duodenum stimulated by preexposure to acid (10 mM HCl for 5 min, $n = 3$). Thus, preventing exposure of guinea pig duodenum to acid by inhibition of gastric secretion induced sensitivity to exogenous PGE_2 , whereas exposure of cat duodenum to HCl reduced sensitivity to PGE_2 .

Effects of some gut hormones. In vitro studies using amphibian duodenum have shown that GIP and low concentrations of (pancreatic) glucagon stimulate HCO_3^- transport, whereas higher concentration of glucagon are inhibitory and secretin is without effect (5). In cats and guinea pigs, secretin in doses previously shown to stimulate pancreatic HCO_3^- secretion (2) had no effect on duodenal HCO_3^- transport in vivo (Fig. 9). As a test of the responsiveness of preparations, PGE_2 (6 μM) was administered at the end of these experiments. This agent increased HCO_3^- transport and PD in the cat but, as discussed previously, had no effect in the guinea pig.

Effects of glucagon (continuous infusion) and GIP (bolus injection) were determined in the guinea pig. Although insensitive to exogenous PGE_2 , both glucagon and GIP increased duodenal HCO_3^- transport. Infusion of glucagon at a dose of 120 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ produced a transient rise in HCO_3^- transport, but higher doses had either no effect or decreased transport rate (Fig. 10). Essentially similar responses (transient increase at low doses and inhibition at high doses) occurred after a bolus injection of glucagon (4–45 $\mu\text{g}/\text{kg}$) in both guinea pigs ($n = 7$) and cats ($n = 5$). GIP (5 $\mu\text{g}/\text{kg}$ iv) increased HCO_3^- transport in the guinea pig ($P < 0.01$), whereas a higher dose (15 $\mu\text{g}/\text{kg}$) produced no further increase (Fig. 11). The changes in transport rate were not accompanied by changes in PD with either glucagon or GIP.

DISCUSSION

Duodenal mucosa from all the species examined spontaneously alkalinized the luminal bathing solution at a steady basal rate. With the exception of pigs, the segment of duodenum studied was devoid of Brunner's glands and, in every case, of pancreatic and bile secretions. The alkaline secretion thus originated from the duodenal epithelium per se. Titration end points of pH 7.4 and 8.0 were used to maintain a very low luminal H^+ concentration and thus preclude passive diffusion of H^+ from lumen into tissue, contributing to measured rates of alkalinization. Alkaline secretion may then reflect secretion of HCO_3^- (or OH^-) into the lumen or active transport of H^+ from the lumen. Previous demonstrations of Pco_2 levels in the duodenal lumen (devoid of pancreatic or bile

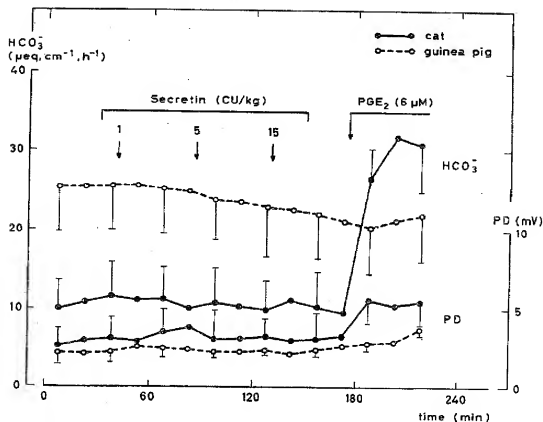


FIG. 9. Secretin (1–15 CU/kg iv) had no effect on duodenal HCO_3^- transport and transepithelial potential difference (PD) in either cat or guinea pig. Although prostaglandin E_2 (PGE_2 , 6 μM) stimulated secretion and increased PD in cat, this agent had no effect in basal secreting guinea pig preparations. Mean values \pm SE are shown ($n = 5$ for cat and $n = 4$ for guinea pig).

HCO_3^-) considerably above those in tissue or blood (10, 14) strongly suggest that secretion of HCO_3^- is the case. This may occur by transport of this ion *per se* or, as discussed for other epithelia (1), be the result of cell membrane transport of H^+ or OH^- combined with counter/cotransport of CO_2 .

There were considerable differences in basal rates of duodenal HCO_3^- secretion among species, although these rates were consistently greater than in the stomach. For example, HCO_3^- output from 1 cm of guinea pig duodenum ($\sim 40 \mu\text{eq/h}$) is as great as that of the entire stomach (8) in animals of the same size and exposed to the same anesthesia. Similarly, much higher duodenal than gastric (5, 9, 17) HCO_3^- rates occur in cats and dogs. These data are analogous to findings with bullfrog *in vitro* preparations in which duodenal HCO_3^- transport (~ 1.0) is much higher than in either gastric antrum (~ 0.45) or fundus ($\sim 0.55 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$) from the same species (3). When rates of HCO_3^- transport in the same animal (cat) were compared, basal and prostaglandin-stimulated transport was also greater (threefold) in the duodenum than in the jejunum (Table 2). In the cat, stimulation of jejunal transport was associated with an increase in PD (lumen negative) as found previously in the dog (25). Higher rates of HCO_3^- transport in duodenum compared with jejunum have also been observed from amphibian intestine *in vitro* (29). The ability of duodenum to secrete HCO_3^- at a high rate is of interest in view of its regular exposure to acid.

Exposure of cat and rat duodenum to exogenous acid (10 mM HCl) resulted in an increase in the rate of HCO_3^- transport. The fall in DNA release after acidification makes it very unlikely that either the increase in

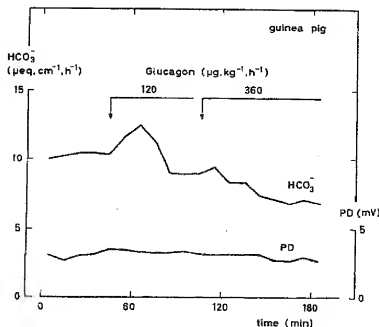


FIG. 10. Glucagon was infused intravenously in guinea pig at doses of 120 and 360 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. Low doses of hormone transiently stimulated HCO_3^- transport in guinea pig duodenum, whereas higher doses were inhibitory. No changes in transepithelial potential difference (PD) occurred in any experiments after administration of glucagon.

HCO_3^- secretion or prostaglandin output was due to mucosal damage (cellular exfoliation). It is thus probable that the increase in HCO_3^- transport afforded adequate protection of the mucosal surface from H^+ .

The appearance of prostaglandin in the lumen and the

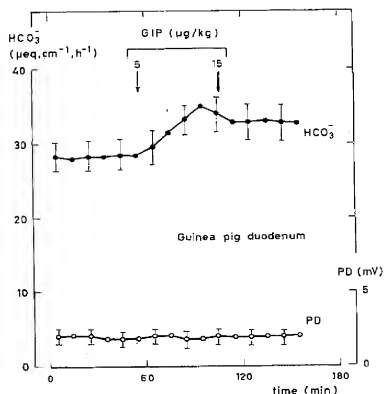


FIG. 11. Gastric inhibitory peptide (GIP) stimulated duodenal HCO_3^- transport in guinea pig after intravenous injection at a dose of 5 $\mu\text{g}/\text{kg}$. No further increase in secretion occurred when dose was raised to 15 $\mu\text{g}/\text{kg}$, and there were no changes in transepithelial electrical potential difference (PD) at either dose. Mean values \pm SE are shown ($n = 6$).

rise in HCO_3^- transport after exposure to acid were inhibited by indomethacin (Fig. 3), suggesting a role of endogenous prostaglandins in mediating the response. There was a time lag between the rise in HCO_3^- and the increase in luminal prostaglandin appearance that may reflect time for diffusion of prostaglandin from tissue to lumen. In contrast to the response to acid, however, stimulation by exogenous PGE_2 in the cat (but not the rat) was associated with a rise in PD. Further, an increase in duodenal HCO_3^- transport after exposure to acid also occurred in animals pretreated with indomethacin, although the profile in the response (sustained in the absence of indomethacin and transient in the presence of this agent) differed. Thus, it is possible that both endogenous E-type prostaglandins and other mediators/mechanisms are involved in mediating stimulation of duodenal HCO_3^- transport in response to acid and that interactions occur. Experiments in amphibian isolated gastric and duodenal mucosa *in vitro* (12) suggest a transient increase in HCO_3^- transport in response to luminal acid mediated by a humoral (nonprostaglandin) mechanism. The design of the present experiments cannot exclude involvement of a nervous reflex in addition to humoral mediation.

Exogenous PGE_2 stimulated epithelial HCO_3^- transport in cat, rat, and dog duodenum but did not affect basal transport in guinea pigs and rabbits. A similar species difference was observed with respect to the action of exogenous acid, which failed to stimulate (basal) duodenal HCO_3^- transport in the guinea pig. The apparent

lack of response to PGE_2 in untreated guinea pigs probably results from high levels of endogenous prostaglandin induced by continuous exposure of the duodenum to acid emptying from the stomach. Thus, PGE_2 stimulated HCO_3^- transport in animals pretreated with inhibitors of tissue prostaglandin synthesis or when a histamine H_2 -antagonist had been used to inhibit gastric acid secretion. A difference between herbivores (continuous feeders) and carnivores (intermittent feeders) has also been observed with respect to stimulation of pancreatic HCO_3^- secretion (2). The lack of response to PGE_2 in guinea pigs pretreated with a high dose (25 mg/kg) of indomethacin may be in accordance with reported effects of this agent on intestinal mucosa unrelated to inhibition of tissue cyclooxygenase activity (32). It was also observed in the present study that exposure of cat duodenum to acid rendered this tissue refractory to exogenous PGE_2 (6 μM) and that inhibitors of endogenous prostaglandin synthesis attenuated basal transport of HCO_3^- in guinea pig duodenum but not in cat. This is further evidence for involvement of endogenous prostaglandins in mediating stimulation of HCO_3^- transport by luminal acid in both herbivores and carnivores.

Secretin is a potent stimulant of pancreatic HCO_3^- secretion (2) but had no effect on duodenal HCO_3^- transport in either guinea pigs or cats. Both glucagon and GIP stimulated HCO_3^- transport. These responses were not accompanied by a rise in PD. Recent studies in the isolated amphibian mucosa have demonstrated that proximal duodenum probably possesses two mechanisms for transport of HCO_3^- (6). One mechanism that may be electrogenic (29) is stimulated by prostaglandins and cAMP and another (electroneutral) is stimulated by glucagon and GIP and inhibited by furosemide. Only the latter is dependent on luminal Cl^- , suggesting occurrence of $\text{Cl}^-/\text{HCO}_3^-$ exchange at the luminal cell membrane, and is thus comparable with HCO_3^- transport by gastric mucosa (5) or lower parts of the small intestine (15, 24, 31). Effects of prostaglandins and hormones *in vitro* are also additive (6). The present finding that GIP (Fig. 11) and glucagon stimulate duodenal HCO_3^- transport in basal secreting guinea pig preparations, in spite of their insensitivity to exogenous PGE_2 (Fig. 9), is also suggestive of additive effects *in vivo*. Properties of HCO_3^- transport by mammalian duodenum *in vivo* thus seem very similar to the metabolism-dependent process previously demonstrated *in vitro*.

With the exception of pigs, Brunner's glands were absent in all segments of duodenum from the species studied. Proximal and distal duodenum in dogs (10, 14) and humans (33) disposes of instilled HCl at about an equal rate, although in both these species Brunner's glands are mainly confined to the proximal portion. Thus, it seems probable that surface epithelium rather than Brunner's glands is responsible for removal of H^+ . Glucagon, crude extracts of duodenal mucosa, and some (but not all) preparations of secretin have previously been reported to increase secretion in duodenal pouches of the Brunner's gland area in the dog (13, 30), whereas aspirin has been reported to be inhibitory (27). These results were interpreted as effects on Brunner's gland secretion. However, it should be stressed that fluid and electrolytes

appearing in a proximal duodenal pouch may originate from surface epithelial cells and/or Brunner's glands. Use of micropuncture techniques would seem necessary to determine whether these glands secrete HCO_3^- and whether such a secretion could be stimulated. The present study as well as previous results in proximal amphibian duodenum (6) suggest that the surface epithelium is the origin of duodenal HCO_3^- secretion affected by glucagon and aspirin, a property previously attributed to Brunner's glands.

Assuming cat duodenum to be a cylinder of 6 mm diameter, acid-stimulated HCO_3^- transport would remove H^+ at a rate of $0.3\text{--}0.4 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$. Similarly, basal secreting guinea pig duodenum (assuming a cylinder of 4 mm diameter) would neutralize intraluminal acid at a rate of $\sim 0.5 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$, and transport in the dog (8-mm-diameter cylinder) stimulated by PGE_2 ($6 \mu\text{M}$) would remove acid at a rate of $\sim 0.4 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$. These figures compare favorably with the rate of loss of H^+ of $0.5\text{--}0.6 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ from acid solutions (100 mM HCl) instilled into canine distal or proximal duodenum (14). The combined data suggest that epithelial HCO_3^- transport alone may account for acid disposal by duodenal mucosa. Previous workers have proposed that neutralization by HCO_3^- of mucosal origin could account for some 50% of H^+ disappearance, with the remainder being lost by diffusion of H^+ into the epithelium (10, 33). It should be noted, however, that HCO_3^- transport was quantified by direct titration in the present study, whereas previous (10, 14) studies have estimated duodenal HCO_3^- transport from luminal PCO_2 . This approach may underestimate the magnitude of HCO_3^- secretion due to loss of CO_2 by absorption into the mucosa. Unlike the stomach (8, 17), duodenum (10, 26) seems to absorb CO_2 relatively rapidly.

In view of the high frequency of duodenal ulceration, mechanisms of duodenal H^+ disposal are important to understand. Although pancreatic and bile secretions provide a major source of HCO_3^- entering the duodenal lumen, it would seem logical that pH at the mucosal surface is of greater significance in relation to mucosal protection than pH of the luminal bulk solution. Structural studies on intestinal mucus gel (23) suggest that its

properties are adequate to provide a boundary of low turbulence at the mucosal surface. Neutralization within this boundary of H^+ diffusing from the lumen should enhance the protective effect of HCO_3^- secreted by the surface epithelium as previously proposed for gastric mucosa (cf. Ref. 6). A pH gradient at the luminal surface of rat duodenum has also recently been demonstrated with pH-sensitive microelectrodes (18), pH in the immediate vicinity of the luminal cell membranes being ≥ 7.0 in spite of that in the luminal bulk solution being as low as 2.0. Stimulation of surface epithelial HCO_3^- transport by prostaglandins enhanced and inhibition by acetazolamide reduced the thickness and alkalinity of the gradient.

A defect in epithelial HCO_3^- transport might thus predispose to duodenal ulceration, whereas stimulation of secretion may provide a therapeutic alternative to inhibition of gastric H^+ secretion with the advantage of maintaining a low (acidic) pH in the stomach. In animals E-type prostaglandins prevent duodenal ulceration induced by hypersecretion of HCl, and they are beneficial in treatment of duodenal ulcer disease in humans. Conversely, cyclooxygenase inhibitors (nonsteroidal anti-inflammatory agents) cause ulceration. In the present study, it was found that PGE_2 increased duodenal epithelial HCO_3^- transport, while indomethacin and aspirin inhibited this transport and/or its stimulation by luminal acid. Interestingly, prostaglandins inhibit pancreatic HCO_3^- secretion (19). Secretin, which is a powerful stimulant of this secretion but without effect on duodenal HCO_3^- transport, has proved to be of little benefit (11) in therapy of duodenal ulceration. Increased duodenal mucus production, facilitating maintenance of the surface pH gradient and increased mucosal blood flow, may also contribute to protection by exogenous and endogenous prostaglandins.

We thank A. Bergman and L. Hughes for excellent technical assistance, Dr. P. Naeser and Dr. M. J. Tucker for histology, and A. S. Gierman for secretarial assistance.

G. Flemström was supported by Swedish Medical Research Council Grants 04X-3515 and 04F-3742.

Received 11 December 1981; accepted in final form 25 May 1982.

REFERENCES

1. BRODSKY, W. A., Z. I. CABANTCHIK, N. DAVIDSON, G. EHRENSPECK, E. M. KINNE-SAFRAN, and R. KINNE. Localization and characterization of transport-related elements in the plasma membrane of turtle bladder epithelial cells. *Biochim. Biophys. Acta* 558: 490-503, 1979.
2. CASS, R. M. Secretory processes in exocrine pancreas. *Proc. Aust. Physiol. Pharmacol. Soc.* 9: 29-42, 1978.
3. FLEMSTRÖM, G. Stimulation of HCO_3^- transport in isolated proximal bullfrog duodenum by prostaglandins. *Am. J. Physiol.* 239 (Gastrointest. Liver Physiol. 2): G198-G203, 1980.
4. FLEMSTRÖM, G., and O. NYLANDER. Stimulation of duodenal epithelial HCO_3^- transport in the guinea pig and cat by luminal prostaglandin E_2 . *Prostaglandins* 21, Suppl.: 47-52, 1981.
5. FLEMSTRÖM, G., and A. GARNER. Gastrointestinal HCO_3^- transport: characteristics and proposed role in acidity regulation and mucosal protection. *Am. J. Physiol.* 242 (Gastrointest. Liver Physiol. 5): G183-G193, 1982.
6. FLEMSTRÖM, G., J. R. HEYLLINGS, and A. GARNER. Gastric and duodenal HCO_3^- transport in vitro: effects of hormones and local transmitters. *Am. J. Physiol.* 242 (Gastrointest. Liver Physiol. 5): G100-G110, 1982.
7. FLOREY, H. W., R. D. WRIGHT, and M. A. JENNINGS. The secretions of the intestine. *Physiol. Rev.* 21: 36-69, 1941.
8. GARNER, A., and G. FLEMSTRÖM. Gastric HCO_3^- secretion in the guinea pig. *Am. J. Physiol.* 234 (Endocrinol. Metab. Gastrointest. Physiol. 3): E535-E541, 1978.
9. GARNER, A., and B. HURST. Alkaline secretion by the canine Heidenhain pouch in response to exogenous acid, some gastrointestinal hormones and prostaglandin. *Adv. Physiol. Sci.* 12: 215-219, 1981.
10. HARMON, J. W., M. WOODS, and N. J. GURLL. Different mechanisms of hydrogen ion removal in stomach and duodenum. *Am. J. Physiol.* 235 (Endocrinol. Metab. Gastrointest. Physiol. 4): E692-E698, 1978.
11. HENN, R. M., S. SELDON, R. A. L. STURDEVANT, J. I. ISENBERG, and M. L. GROSBMAN. Experience with synthetic secretin in the treatment of duodenal ulcer. *Dis. Dig.* 21: 921-926, 1976.
12. HEYLLINGS, J. R., and A. GARNER. Influence of luminal acidification

- on bicarbonate transport by gastric and duodenal isolated mucosae. *Prostaglandins* 21, Suppl.: 67-71, 1981.
13. HIMAL, H. S., F. MOQTADERI, D. A. DREILING, A. E. KARK, AND J. RUDICK. Glucagon stimulates Brunner's gland secretion. *Nature London* 228: 767-768, 1970.
 14. HIMAL, H. S., H. W. WERTHER, M. L. CHAPMAN, H. D. JANOWITZ, AND J. RUDICK. Acid absorption in the canine duodenum. *Ann. Surg.* 185: 481-487, 1977.
 15. HUBEL, K. A. Bicarbonate secretion in rat ileum and its dependence on intraluminal chloride. *Am. J. Physiol.* 213: 1409-1413, 1967.
 16. JAFFE, B. M., H. R. BEHRMAN, AND C. N. PARKER. Radioimmunoassay of prostaglandins E, A and F in human plasma. *J. Clin. Invest.* 52: 398-406, 1973.
 17. KAUFFMAN, G. L., AND J. H. STEINBACH. Gastric bicarbonate secretion: effect of pH and topical 16-16 dimethyl prostaglandin E₂. *Surgery* 89: 324-328, 1981.
 18. KIVILAKSO, E., AND G. FLEMSTRÖM. HCO₃⁻ secretion and pH gradient across the surface mucus gel in rat duodenum (Abstract). *Gastroenterology* 82: 1101, 1982.
 19. KONTUREK, S. J., J. TASLER, J. JAWOREK, M. CIESZKOWSKI, AND W. PAWLAK. Prostacyclin inhibits pancreatic secretion. *Am. J. Physiol.* 238 (Gastrointest. Liver Physiol. 1): G531-G536, 1980.
 20. LEON, S. A., A. GREEN, M. J. YAKOS, AND B. SHAPIRO. Radioimmunoassay for nanogram quantities of DNA. *J. Immunol. Methods* 9: 157-164, 1976.
 21. LUCAS, M. L., J. A. BLAIR, B. T. COOPER, AND W. T. TREVOR. Relationship of the acid micro-climate in rat and human intestine to malabsorption. *Biochem. Soc. Trans.* 4: 154-156, 1976.
 22. MANN, F. C., J. P. FOSTER, AND S. D. BRIMHALL. The relation of the common bile duct to the pancreatic duct in common domestic and laboratory animals. *J. Lab. Clin. Med.* 5: 203-206, 1920.
 23. MANTLE, M., D. MANTLE, AND A. ALLEN. Polymeric structure of pig small-intestinal mucus glycoprotein. *Biochem. J.* 195: 277-285, 1981.
 24. POWELL, D. W., S. J. MALAWER, AND G. R. PLOTKIN. Secretion of electrolytes and water by the guinea pig small intestine. *Am. J. Physiol.* 215: 1228-1233, 1968.
 25. READ, N. W., G. J. KREIS, R. M. BARKLEY, S. G. MORAWSKI, AND J. S. FORDTRAN. Spontaneous secretion from the dog small intestine in vivo. *J. Lab. Clin. Med.* 93: 381-389, 1979.
 26. RUNE, S. J., AND F. W. HENRIKSEN. Carbon dioxide tensions in the proximal part of the canine gastrointestinal tract. *Gastroenterology* 56: 758-762, 1969.
 27. RUSSELL, T. R., AND R. S. JONES. The effect of aspirin on duodenal secretion. *Proc. Soc. Exp. Biol. Med.* 145: 967-969, 1974.
 28. SCHLEPPENT, W., D. LUCKE, S. ANURAS, AND D. CHRISTENSEN. Carcinoma of the papilla of Vater. A review of fifty-seven cases. *Am. J. Surg.* 135: 763-770, 1978.
 29. SIMSON, J. N. L., A. MERHAV, AND W. SILEN. Alkaline secretion by amphibian duodenum. I. General characteristics. *Am. J. Physiol.* 240 (Gastrointest. Liver Physiol. 3): G401-G408, 1981.
 30. STENING, G. F., AND M. I. GROSSMAN. Hormonal control of Brunner's glands. *Gastroenterology* 55: 1047-1052, 1969.
 31. TURNBERG, L. A., F. A. BIERBERDORP, S. G. MORAWSKI, AND J. S. FORDTRAN. Interrelationships of chloride, bicarbonate, sodium, and hydrogen transport in the human ileum. *J. Clin. Invest.* 49: 557-568, 1970.
 32. WHITTLE, B. J. R. Temporal relationship between cyclooxygenase inhibition, as measured by prostacyclin biosynthesis, and gastrointestinal damage induced by indomethacin in the rat. *Gastroenterology* 80: 94-98, 1981.
 33. WINSHIP, D. H., AND J. R. ROBINSON. Acid loss in human duodenum. *Gastroenterology* 66: 181-188, 1974.



**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT E

GOODMAN & GILMAN's The PHARMACOLOGICAL BASIS OF THERAPEUTICS

Ninth Edition

McGraw-Hill
HEALTH PROFESSIONS DIVISION

New York St. Louis San Francisco Auckland Bogotá Caracas Lisbon London Madrid
Mexico City Milan Montreal New Delhi San Juan Singapore Sydney Tokyo Toronto

PHARMACODYNAMICS

Mechanisms of Drug Action and the Relationship
Between Drug Concentration and Effect

Elliott M. Ross

This chapter provides an introduction of the concept of receptors, the structural and functional families of receptors, and the interplay between the diverse signaling pathways activated by receptor occupancy. These introductory concepts are amplified in subsequent chapters detailing the structure and function of receptors for individual drug groups. The latter half of the chapter describes the historical development of receptor theory and presents means for quantifying receptor activation by agonists and blockade by antagonists. The functional relevance of partial agonists and inverse antagonists also is described as a prelude to the intentional development of mechanistically diverse drugs via classical or new combinatorial strategies.

Pharmacodynamics can be defined as the study of the biochemical and physiological effects of drugs and their mechanisms of action. The objectives of the analysis of drug action are to delineate the chemical or physical interactions between drug and target cell and to characterize the full sequence and scope of actions of each drug. Such a complete analysis provides the basis for both the rational therapeutic use of a drug and the design of new and superior therapeutic agents. Basic research in pharmacodynamics also provides fundamental insights into biochemical and physiological regulation.

MECHANISMS OF DRUG ACTION

The effects of most drugs result from their interaction with macromolecular components of the organism. These interactions alter the function of the pertinent component and thereby initiate the biochemical and physiological changes that are characteristic of the response to the drug. This concept—now obvious—had its origins in the experimental work of Ehrlich and Langley during the late nineteenth and early twentieth centuries. Ehrlich was struck by the high degree of chemical specificity for the antiparasitic and toxic effects of a variety of synthetic organic chemicals. Langley noted the ability of the South American arrow poison, curare, to inhibit the contraction of skeletal muscles caused by nicotine; however, the tissue remained responsive to direct electrical stimulation. The term *receptor* was

coined to denote the component of the organism with which the chemical agent was presumed to interact.

The statement that the receptor for a drug can be any functional macromolecular component of the organism has several fundamental corollaries. One is that a drug potentially is capable of altering the rate at which any bodily function proceeds. Another is that drugs do not create effects, but instead modulate functions.

Drug Receptors

At least from a numerical standpoint, proteins form the most important class of drug receptors. Examples are the receptors for hormones, growth factors, and neurotransmitters, the enzymes of crucial metabolic or regulatory pathways (e.g., dihydrofolate reductase, acetylcholinesterase), proteins involved in transport processes (e.g., Na^+ , K^+ -ATPase), or proteins that serve structural roles (e.g., tubulin). Specific binding properties of other cellular constituents also can be exploited. Thus, nucleic acids are important drug receptors, particularly for cancer chemotherapeutic agents.

A particularly important group of drug receptors are proteins that normally serve as receptors for endogenous regulatory ligands (e.g., hormones, neurotransmitters). Many drugs act on such physiological receptors and are often particularly selective, because physiological receptors are specialized to recognize and respond to individual signal-

ing molecules with great selectivity. Drugs that bind to physiological receptors and mimic the effects of the endogenous regulatory compounds are termed *agonists*. Other drugs bind to receptors and do not mimic, but interfere with, the binding of the endogenous agonist. Such compounds, which are themselves devoid of intrinsic regulatory activity, but which produce effects by inhibiting the action of an agonist (e.g., by competition for agonist binding sites), are termed *antagonists*. There are additional subtleties to drug classification. Thus, agents that are only partly as effective as agonists are termed *partial agonists*, and those that stabilize the receptor from undergoing productive agonist-independent conformational changes are termed *negative antagonists* or *inverse agonists*. (See below, "Quantitation of Drug-Receptor Interactions and Elicited Response.")

The binding of drugs to receptors can involve all known types of interactions—ionic, hydrogen bonding, hydrophobic, van der Waals, and covalent. In most interactions between drugs and receptors, it is likely that bonds of multiple types are important. If binding is covalent, the duration of drug action is frequently, but not necessarily, prolonged. Noncovalent interactions of high affinity also may appear to be essentially irreversible.

Structure-Activity Relationship and Drug Design. Both the affinity of a drug for its receptor and its intrinsic activity are determined by its chemical structure. This relationship is frequently quite stringent. Relatively minor modifications in the drug molecule may result in major changes in pharmacological properties.

Exploitation of structure-activity relationships has on many occasions led to the synthesis of valuable therapeutic agents. Because changes in molecular configuration need not alter all actions and effects of a drug equally, it is sometimes possible to develop a congener with a more favorable ratio of therapeutic to toxic effects, enhanced selectivity among different cells or tissues, or more acceptable secondary characteristics than those of the parent drug. Therapeutically useful antagonists of hormones or neurotransmitters have been developed by chemical modification of the structure of the physiological agonist. Minor modifications of structure also can have profound effects on the pharmacokinetic properties of drugs.

Given adequate information about both the molecular structures and the pharmacological activities of a relatively large group of congeners, it should be possible to identify those properties that are required for optimal action at the receptor—size, shape, the position and orientation of charged groups or hydrogen bond donors, and so on. Recent advances in computational chemistry, structural analysis of organic compounds, and the biochemical measurement of the pri-

mary actions of drugs at their receptors have enriched the quantitation of structure-activity relationships and its use in drug design (Kantiz, 1992; Schreiber, 1992). By accurately and quantitatively correlating the pharmacological activities of multiple drugs with their molecular structures—their overall shapes and the locations and orientations of chemically interactive groups on their surfaces—it is possible to model accurately the structure of the binding site on the receptor. Such detailed models allow the informed design of improved congeners or the *de novo* design of novel compounds that can bind to the receptor with improved selectivity, affinity, or regulatory effect. Such considerations also allow computerized searching of large chemical libraries for diverse compounds that, because of their overall three-dimensional structures, should act upon the receptor of interest. Similar structure-based approaches also can be used to improve pharmacokinetic properties of drugs (see Chapter 1).

Recent advances using the structures of receptors and of drug-receptor complexes, determined at atomic resolution by X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy, are even more helpful in the initial design of ligands. In cases where the structure of the entire receptor is unknown, it is often possible to determine the conformation of the bound drug, thereby providing a mirror image of the receptor's binding site. The ability to clone and express cDNAs that encode less abundant regulatory proteins and increasing success in the crystallization of membrane-bound proteins offer great promise for drug design based on a detailed knowledge of the drug binding site and the effect of drug binding on receptor structure.

Ironically, advances in molecular biology that contribute to structure-motivated drug design also have spawned powerful but entirely random searches for new drugs. In this approach, huge libraries of randomly synthesized chemicals are generated either by synthetic chemistry or by genetically engineered microbes. A library then is screened for pharmacologically active agents using mammalian cells or microorganisms that have been engineered to express the receptor of therapeutic interest and the associated biochemical machinery necessary for detection of the receptor's response. Active compounds initially discovered by such random screens then can be modified and improved using knowledge of their structure-function relationships.

Cellular Sites of Drug Action. The sites at which a drug acts and the extent of its action are determined by the localization and functional capacity of the specific receptors with which the drug interacts and the concentration of drug to which the receptor is exposed. Selective localization of drug action within the organism is therefore not necessarily dependent upon selective distribution of the drug.

If a drug acts on a receptor that serves functions common to most cells, its effects will be widespread. If the function is a vital one, the drug will be particularly difficult or dangerous to use. Nevertheless, such a drug may be clinically important. Digitalis glycosides, important in the treatment of heart failure, are potent inhibitors of an ion transport process that is vital to most cells. As such, they can cause widespread toxicity, and their margin of safety is dangerously low. Other examples could be cited, particularly in the area of cancer chemotherapy.

If a drug interacts with receptors that are unique to only a few types of differentiated cells, its effects are more specific. Hypothetically, the ideal drug would cause its therapeutic effect by such an action. Side effects would be minimized, but toxicity might not be. If the differentiated function were a vital one, this type of drug also could be very dangerous. Some of the most lethal chemical agents known (e.g., botulinus toxin) show such specificity and toxicity. Note

also that, even if the primary action of a drug is localized, the physiological effects of the drug may be widespread.

Physiological Receptors

The term *receptor* has been used operationally to denote any cellular macromolecule to which a drug binds to initiate its effects. Among the most important drug receptors are cellular proteins whose normal function is to act as receptors for endogenous regulatory ligands—particularly hormones, growth factors, neurotransmitters, and autacoids. The function of such physiological receptors consists of binding the appropriate ligand and, in response, propagating its regulatory signal in the target cell.

Identification of the two functions of a receptor, ligand-binding and message propagation, led to speculation on the existence of functional domains within the receptor: a *ligand-binding domain* and an *effector domain*. The evolution of different receptors for diverse ligands that act by similar biochemical mechanisms, on the one hand, and of multiple receptors for a single ligand that act by unrelated mechanisms, on the other, supports this concept. Indeed, elucidation of the structure of a well-characterized receptor often allows the identification of these specialized domains within the primary amino acid sequence or the three-dimensional structure of the protein.

The regulatory actions of a receptor may be exerted directly on its cellular target(s), *effector protein(s)*, or may be conveyed to cellular targets by intermediary cellular molecules, *transducers*. The receptor, its cellular target, and any intermediary molecules are referred to as a *receptor-effector system* or *signal transduction pathway*. Even the effector protein may not be the ultimate cellular component affected but may synthesize or release another signaling molecule, usually a small metabolite or ion, known as a *second messenger*.

Receptors (and their associated effector and transducer proteins) also act as integrators of extracellular information as they coordinate signals from multiple ligands with each other and with the metabolic activities of the cell (see below). This integrative function is particularly evident when one considers that the different receptors for scores of chemically unrelated ligands utilize relatively few biochemical mechanisms to exert their regulatory functions and that even these few pathways may share common elements.

An important property of physiological receptors, which also makes them excellent targets for drugs, is that they act catalytically and hence are biochemical signal amplifiers. The catalytic nature of receptors is obvious when

the receptor itself is an enzyme, but all known physiological receptors are formally catalysts. For example, when a single ligand molecule binds to a receptor that is an ion channel and opens it, many ions flow through the channel. Similarly, a single steroid hormone molecule binds to its receptor and initiates the transcription of many copies of specific mRNAs, which in turn can give rise to multiple copies of a single protein.

Physiological Receptors: Structural and Functional Families. The last decade has witnessed both an explosion in our appreciation of the number of physiological receptors and, in parallel, the development of our understanding of the fundamental structural motifs and biochemical mechanisms that characterize them. Molecular cloning has identified both completely novel receptors (and their regulatory ligands) and numerous isoforms of previously known receptors. There now exist data banks devoted exclusively to structures of a single class of receptors. Members of various classes of receptors and many of the associated transducer and effector proteins have been purified, and their mechanisms of action are understood in considerable biochemical detail. Receptors, transducers, and effectors can be expressed via molecular genetic strategies and studied in cultured cells. Alternatively, they can be expressed in large amounts in cells of convenience (bacteria, yeast, etc.) to facilitate their purification.

Receptors for physiological regulatory molecules can be assigned to several functional families whose members share both common mechanisms of action and, with the exception of the protein kinases, strikingly homologous structures. This homology and the results of extensive biochemical and genetic studies have advanced the two-domain concept of receptor structure beyond conjecture. For each receptor family, there is now at least a rudimentary understanding of the structures of ligand-binding domains and effector domains and of how agonist binding influences the regulatory activity of the receptor. The small number of mechanisms and structural formats has profound implications for both the integration of signals from receptors for diverse ligands and the regulation of receptors and receptor-effector systems by the target cell. It also facilitates a clear overview of signaling pathways that is generally applicable to diverse tissues, disease states, and therapeutic agents. Figure 2-1 provides a schematic diagram of various receptor families and their transducer and effector molecules.

Receptors as Enzymes: Receptor Protein Kinases. Receptors for peptide hormones that regulate growth, differentiation, and development (and in some cases acute metabolic activity) are frequently plasma membrane-bound protein kinases that act by phosphorylating

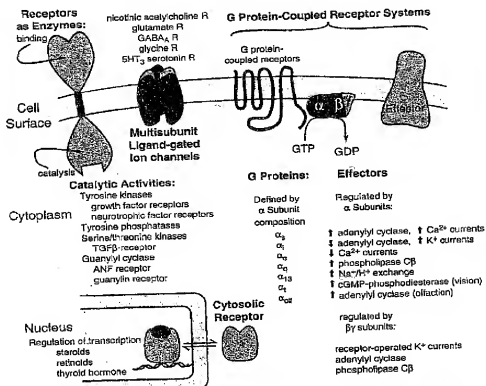


Figure 2-1. Structural motifs of physiological receptors and their relationships to signaling pathways.

Schematic diagram of the diversity of mechanisms for control of cell function by receptors for endogenous agents acting via the cell surface or in the nucleus.

target proteins (Fand *et al.*, 1993). These targets may be enzymes (including other kinases), regulatory proteins, or structural proteins, and phosphorylation may either alter their individual activities or influence their interactions with other regulatory proteins or effectors. Many receptor protein kinases phosphorylate specific tyrosine residues on their target proteins, but a few phosphorylate serine or threonine residues. Receptors that are tyrosine protein kinases include those receptors for insulin, epidermal growth factor, platelet-derived growth factor, and certain lymphokines. Receptors that are serine/threonine protein kinases include the isoforms of receptors for transforming growth factor β . These receptor kinases are assembled from definable domains that are distinguished in part by their location relative to the plasma membrane. The extracellular, hormone-binding domain is connected to an intracellular protein kinase catalytic domain by a relatively short sequence of hydrophobic amino acid residues that cross the plasma membrane; some members of the receptor kinase families are monomers, and others are assembled from nonidentical subunits. Because of the homology among the protein kinase domains in this family, active chimeric receptors have been constructed from different intracellular (catalytic) and extracellular (hormone binding) regions; these chimeras display specificities for hormones and substrates that reflect their parentage. A substantial literature suggests that oligomerization of tyrosine kinase receptors is a critical event in their activation and alteration of cellular functions.

Another family of receptors that are functionally protein kinases contains a modification of the structures described above. Some pro-

tein kinase-associated receptors lack the intracellular enzymatic domains but, in response to agonists, bind and/or activate independent membrane-embedded or cytosolic protein kinases. Receptors of this family that elicit tyrosine phosphorylation include several receptors for neurotrophic peptides and the multisubunit antigen receptors on T and B lymphocytes. There is evidence that the antigen receptors also involve tyrosine protein phosphatases in their cellular regulatory activity, and it is plausible that other receptors that apparently lack cytoplasmic effector domains may recruit still other effector proteins.

Receptors with Other Enzymatic Activity. The domain structure just described for cell surface protein kinases is varied in other receptors to utilize other signaling outputs. In the receptors for atrial natriuretic peptides and the peptide guanylin, the intracellular domain is not a protein kinase but is a guanylyl cyclase, which synthesizes the second messenger, cyclic GMP (Chinkers and Garbers, 1991). Receptors with guanylyl cyclase activity also serve as pheromone receptors in invertebrates. There may be other variations on this transmembrane topology.

A family of protein tyrosine phosphatases has extracellular domains with a sequence reminiscent of cellular adhesion molecules (Walton and Dixon, 1993). Although the extracellular ligands for these phosphatases are not yet known, the importance of their enzymatic activity has been demonstrated through genetic and biochemical experiments in multiple cell types.

Ion Channels. Receptors for several neurotransmitters form agonist-regulated, ion-selective channels in the plasma membrane, termed **ligand-gated ion channels**, which convey their signals by

altering the cell's membrane potential or ionic composition (Hall, 1992). This group includes the nicotinic cholinergic receptor, the GABA_A receptor for gamma-aminobutyric acid, and receptors for glutamate, aspartate, and glycine (see Chapters 7, 9, and 12). They are all multi-subunit proteins with each subunit predicted to span the plasma membrane. The mode of association of the subunits appears to form the channel. Of therapeutic importance is that allosteric modifiers of channel gating can have profound pharmacological effects; for example, the benzodiazepines allosterically enhance Cl⁻ transport through the GABA_A receptor (see Chapters 17 and 18).

G Protein-Coupled Receptors. Many receptors in the plasma membrane regulate distinct effector proteins through the mediation of a group of GTP-binding proteins known as G proteins (Ross, 1992). Receptors for biogenic amines, eicosanoids, and many peptide hormones utilize G protein-coupled receptors. Receptors in this group act by facilitating the binding of GTP to specific G proteins. GTP binding activates the G protein, such that it in turn can regulate the activity of specific effectors. The effectors include enzymes such as adenylyl cyclase and phospholipases A₂, C, and D; channels that are specific for Ca²⁺, K⁺, or Na⁺; and certain transport proteins. An individual cell may express multiple G proteins; each of these may respond to several different receptors and regulate several different effectors with a characteristic pattern of selectivities. G protein-linked receptors and the G proteins themselves both constitute families of homologous proteins. The receptors are hydrophobic proteins that span the plasma membrane in seven α -helical segments. The binding site for small ligands can be a pocket within the bundle of membrane-spanning helices, but a substantial extracellular domain is important for the binding of negatively charged ligands, such as glutamate, or of peptide hormones. The receptors interact with G proteins at their cytoplasmic face, and it has been possible to define specific regions in G protein-coupled receptor structures that are responsible for regulation of and selectivity among the different G proteins.

The G proteins are bound to the inner face of the plasma membrane. They are heterotrimeric molecules (subunits are designated α , β , and γ), and their classification is based on the identity of their distinct α subunits. These polypeptides have highly homologous guanine nucleotide binding domains and have distinct domains for interactions with receptors and effectors. When the system is inactive, GDP is bound to the α subunit (Figure 2-2). An agonist-receptor complex facilitates GTP binding to the α subunit in part by promoting the dissociation of bound GDP. Binding of GTP activates the α subunit, and the α -GTP subunit is then thought to dissociate from the $\beta\gamma$ subunits and interact with a membrane-bound effector. The $\beta\gamma$ subunits also can interact with and influence effector activity independent of or in parallel with α -GTP subunit effects (Clapham and Nee, 1993). Termination of signal transmission results from hydrolysis of GTP to GDP by a GTPase that is intrinsic to the α subunit and the resulting reassociation of α and $\beta\gamma$ subunits. Thus, G proteins serve as regulated molecular switches capable of eliciting bifurcating signals through α and $\beta\gamma$ subunit effects. The switch is turned on by the receptor and turns itself off within a few seconds, a time sufficient for considerable amplification of signal transmission.

If a cell has several receptors that regulate a common effector or that utilize a common transducer, many individual extracellular signals can be integrated to yield a cumulative intracellular signal. G protein-coupled receptor-effector systems provide impressive examples of such integration, as well as the ability to direct a signal to divergent cellular effectors (see Figure 2-3 and related discussion later in this chapter). It is not unusual for several receptors in an individual cell to activate a single G protein; several agonists may stim-

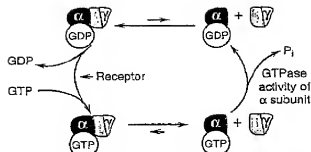


Figure 2-2. The regulatory cycles involved in G protein-mediated signal transduction.

GTP-binding protein activation of effectors is regulated simultaneously by a GTPase cycle and subunit association/dissociation cycle. The GTP-liganded α subunit activates some processes exclusively, and release of $\beta\gamma$ subunits upon activation of G_o allows for regulation by $\beta\gamma$ subunits of shared or distinct effectors.

ulate adenylyl cyclase through a single G protein known as G_s. One receptor also can regulate more than one G protein; as individual thrombin receptor can cause the inhibition of adenylyl cyclase and the activation of phospholipase C by interactions with at least two different G proteins. Similarly, one G protein can regulate several effectors. Thus, the receptor-G protein-effector systems are complex networks of convergent and divergent interactions that permit extraordinarily versatile regulation of cell function (Ross, 1992).

Transcription Factors. Receptors for steroid hormones, thyroid hormone, vitamin D, and the retinoids are soluble DNA-binding proteins that regulate the transcription of specific genes (Evans, 1988; Mangelndorf *et al.*, 1994). They are part of a larger family of transcription factors whose members may be regulated by phosphorylation, association with other protein factors, or by binding to metabolites or cellular regulatory ligands. These receptors act as dimers, some as homodimers and some as heterodimers, with homologous cellular proteins, but may be regulated by higher order oligomerization with other regulating molecules. They provide striking examples of conservation of structure and mechanism, in part because they are assembled as three largely independent domains. The region nearest the carboxyl terminus binds hormone and serves a negative regulatory role; that is, removal of this domain leaves a constitutively active fragment that may be nearly as effective in regulating transcription as is the intact hormone-liganded receptor. Hormone binding presumably also relieves this inhibitory constraint. The central region of the receptor mediates binding to specific sites on nuclear DNA to activate or inhibit transcription of the nearby gene. These regulatory sites in DNA are likewise receptor-specific: the sequence of a "glucocorticoid-responsive element," with only slight variation, is associated with each glucocorticoid-responsive gene. The function of the amino-terminal region of the receptor is less well defined, but its loss decreases the receptor's regulatory activity. The DNA-binding receptors form a homologous family, sharing quite similar amino acid sequences in their DNA-binding domains, less similarity in their hormone-binding domains, and negligible similarity at their amino termini. The activity of each domain is stereotyped and largely independent, a phenomenon best demonstrated by the construction of chimeric receptors that reflect the hormone binding or DNA regula-

tory activity characteristic of the "parent" receptor contributing that domain.

Cytoplasmic Second Messengers. Physiological signals also are integrated within the cell as a result of interactions between second messenger pathways. There are relatively few recognized cytoplasmic second messengers. Thus, their synthesis or release reflects the activities of many pathways. Second messengers influence each other both directly, by altering the other's metabolism, and indirectly, by sharing intracellular targets. This superficially confusing pattern of regulatory pathways allows the cell to respond to agonists, singly or in combination, with an integrated array of cytoplasmic second messengers and responses (see Figure 2-3).

Cyclic AMP, the first recognized second messenger, is synthesized by adenylyl cyclase in response to activation of many receptors; stimulation is mediated by G_s and inhibition by one or more closely related G proteins termed G_i 's. There exist at least ten tissue-specific adenylyl cyclase isozymes, each with its unique pattern of regulatory responses (Tausig *et al.*, 1994). Several adenylyl cyclase isozymes are stimulated by the G protein $\beta\gamma$ subunits, which allows activation of G proteins other than G_s to inhibit cyclase activity. Other isozymes are stimulated by $G\beta\gamma$ subunits, but this stimulation is dependent upon concurrent stimulation by the α subunit of G_s . Still other isozymes are stimulated by Ca^{2+} or Ca^{2+} -calmodulin complexes. Finally, each of the isozymes has its own pattern of enhancement or attenuation by phosphorylation or other reg-

ulatory influences, providing a broad array of regulatory features to the target cells where these isoforms are expressed.

The hydrolysis of cyclic AMP is catalyzed by several phosphodiesterases (Strada and Hidaka, 1992), and the extrusion of cyclic AMP from the cell is accomplished by at least one regulated active transport system. In most cases, cyclic AMP functions by activating cyclic AMP-dependent protein kinases, which regulate numerous intracellular proteins by catalyzing their phosphorylation (see Edelman *et al.*, 1987). In at least one case, olfaction, cyclic AMP mediates signaling by direct binding to and allosteric activation of ligand-activated Na^+ channels.

The cytoplasmic concentration of Ca^{2+} , another ubiquitous second messenger, is controlled both by regulation of several different Ca^{2+} -specific channels in the plasma membrane and by its release from intracellular storage sites. Ca^{2+} channels can be opened by electrical depolarization, by phosphorylation by a cyclic AMP-dependent protein kinase, by G_s , by K^+ , or by Ca^{2+} itself. Opening can be inhibited by other G proteins (G_i and G_o). One channel may respond to several of these inputs.

Release of Ca^{2+} from intracellular stores is mediated by yet another second messenger, inositol 1,4,5-trisphosphate (IP_3). IP_3 is the product of the hydrolysis of the membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2); this reaction is catalyzed by a phospholipase C (PLC; see Figure 2-3). Three families of PLCs, each with several homologous members, respond to three distinct signaling pathways. The PLC- β 's (there is no PLC- α) are stimulated

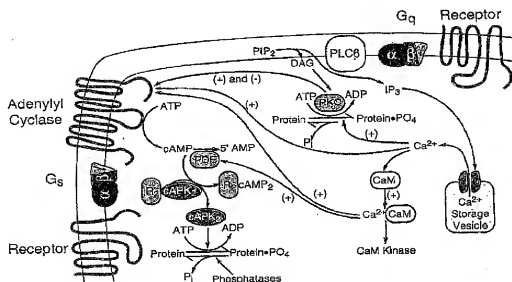


Figure 2-3. Interactions between the second messengers cyclic AMP and Ca^{2+} .

Generation of second messengers, cyclic AMP (cAMP) and Ca^{2+} , permits distribution of cell-surface regulatory input within the cell interior, amplification of the initial signal, and opportunities for synergistic or antagonistic regulation of other signaling pathways. PIP_2 , phosphatidylinositol 4,5, bisphosphate; DAG, diacylglycerol; IP_3 , 1,4,5 inositol trisphosphate; CaM, calmodulin; R_s , regulatory subunits of cyclic AMP-dependent protein kinase, which bind cyclic AMP; cAMPK, catalytic subunits of cyclic AMP-dependent protein kinase; PKC, protein kinase C, activated by DAG and Ca^{2+} .

by the G_q family of G proteins and, for some members, by the G protein $\beta\gamma$ subunits. The PLC- γ 's are activated by phosphorylation on tyrosine residues and are thus activated by cell surface receptor-activated tyrosine kinase cascades. It is not known what regulates the PLC- δ 's (Rhee and Choi, 1992).

Ca^{2+} regulates cellular activity by interaction with several protein mediators; salient examples are protein kinase C and calmodulin (Berridge, 1993). Protein kinase C, like the cyclic AMP-dependent protein kinase, has many substrates, including several proteins that are involved in other signaling systems. The activation of protein kinase C by Ca^{2+} is potentiated by diacylglycerol, the other second messenger product of the phospholipase C-catalyzed reaction that liberates IP_3 . The scope of calmodulin's regulatory activity also is broad. Thus, with reference only to the examples of cyclic AMP and Ca^{2+} , one can appreciate the complexity of integration of cellular signaling systems as reflected in Figure 2-3.

Regulation of Receptors

It is important to recognize that receptors not only initiate regulation of physiological and biochemical function but also are themselves subject to many regulatory and homeostatic controls. For example, continued stimulation of cells with agonists generally results in a state of *desensitization* (also referred to as *refractoriness* or *down regulation*), such that the effect that follows continued or subsequent exposure to the same concentration of drug is diminished (Figure 2-4). This phenomenon can become very important in therapeutic situations; an example is attenuated response to the repeated use of β -adrenergic agonists as bronchodilators for the treatment of asthma (see Chapter 10).

Multiple mechanisms account for desensitization of different types (see Perkins *et al.*, 1990). In some cases, only the signal from the stimulated receptor becomes attenuated, a process known as *homologous desensitization*. This may involve covalent modification (e.g., phosphorylation) of the receptor, the destruction of the receptor, or its relocation within the cell. Synthesis of receptors also is subject to feedback regulation. In other situations, receptors for different hormones that act on a single signaling pathway may all become less effective when only one is continuously stimulated. Such *heterologous desensitization* may result either from modification of each receptor by a common feedback mechanism or from effects exerted at some common point in the effector pathway distal to the receptor itself. Mechanisms involved in

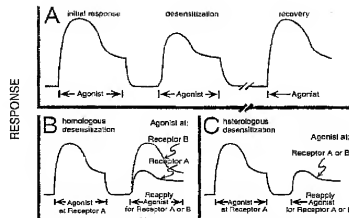


Figure 2-4. Desensitization in response to an agonist.

A. Upon exposure to an agonist, the initial response usually peaks and then decreases to approach some tonic level, elevated but below the maximum. If the drug is removed for a brief period, the state of *desensitization* is maintained such that a second addition of agonist also provokes a diminished response. Removal of the drug for a more extended period allows the cell to "reset" its capacity to respond, and *recovery* of response usually is complete. (B and C) Desensitization may be *homologous* (B), affecting responses elicited only by the stimulated receptor, or *heterologous* (C), acting on several receptors or on a pathway that is common to many receptors.

homologous and heterologous desensitization of specific receptors and signaling pathways will be discussed in greater detail in later chapters related to individual receptor families.

Predictably, hyperreactivity or supersensitivity to receptor agonists also frequently is observed to follow reduction in the chronic level of receptor stimulation. Situations of this type can result, for example, from the long-term administration of β -adrenergic antagonists such as propranolol (see Chapter 10). In at least some cases, supersensitivity may result from the synthesis of additional receptors.

Diseases Resulting from Receptor Malfunction. In addition to variability among individuals in their responses to drugs (see Chapter 3), several definable diseases arise from disorders in receptors or receptor-effector systems. The loss of a receptor in a highly specialized signaling system may cause a relatively limited phenotypic disorder, such as the genetic deficiency of the androgen receptor in the testicular feminization syndrome (Griffin and Wilson, 1989). Deficiencies of more widely used signaling systems have a broader spectrum of effects, as are seen in myasthenia gravis or some forms of insulin-resistant diabetes mellitus, which result from autoimmune depletion of nicotinic cholinergic receptors (see Chapter 9) or insulin receptors (see Chapter 60), respectively. A lesion in a component of a signaling pathway that is used by many receptors can cause a generalized endocrinopathy. Heterozygous deficiency of G_s , the G pro-

tein that activates adenylyl cyclase in all cells, causes multiple endocrine disorders; the disease is termed *pseudohypoparathyroidism type 1a* (Spiegel, 1989). Homozygous deficiency in G_s would presumably be lethal.

The expression of aberrant or ectopic receptors, effectors, or coupling proteins potentially can lead to supersensitivity, subsensitivity, or other untoward responses. Among the most interesting and significant events is the appearance of aberrant receptors as products of *oncogenes*, which transform otherwise normal cells into malignant cells. Virtually any type of signaling system may have oncogenic potential. The *erbA* oncogene product is an altered form of a receptor for thyroid hormone, constitutively active because of the loss of its ligand-binding domain (Evans, 1988). The *ras* and *erbB* oncogene products are activated, uncontrolled forms of the receptors for insulin and epidermal growth factor, both known to enhance cellular proliferation (Yarden and Ullrich, 1988). The *mas* oncogene product (Young *et al.*, 1986) is a G protein-coupled receptor, probably the receptor for a peptide hormone. Constitutive activation of G protein-coupled receptors due to subtle mutations in receptor structure has been shown to give rise to retinitis pigmentosa, precocious puberty, and malignant hyperthyroidism (reviewed in Clapham, 1993). G proteins can themselves be oncogenic when either overexpressed or constitutively activated by mutation (Lyons *et al.*, 1990).

Detection and Characterization of Receptors by Ligand-Binding Assays. Much of the success in the identification, purification, and characterization of receptors reflects their quantitative measurement according to the binding of highly specific radioactive ligands (Limbird, 1995). Binding assays permit the direct study of the drug-binding properties of receptors and reduce the need to rely on inferences derived from the measurement of distal physiological responses. Such inferences are confounded if the observed response lies many steps removed from the receptor and may be compromised by changes at any site in the pathway leading from receptor to ultimate effector. Direct measurement of receptors and analysis of their ligand-binding properties and mechanisms of action have led to our understanding of the mechanisms of receptor action, pathophysiology, and therapeutic effects. For example, we now appreciate the molecular causes of many diseases of receptor malfunction (see above).

Classification of Receptors and Drug Effects

Traditionally, drug receptors have been identified and classified primarily on the basis of the effect and relative potency of selective agonists and antagonists. For example, the effects of acetylcholine that are mimicked by the alkaloid muscarine and that are selectively antagonized by atropine are termed *muscarinic effects*. Other effects of acetylcholine that are mimicked by nicotine and that are not readily antagonized by atropine but are selectively blocked by other agents (e.g., tubocurarine) are described as *nicotinic effects*. By extension, these two types of cholinergic effects are said to be mediated by muscarinic or nicotinic receptors. Such classification of receptors results in an internally consistent scheme that supports the view that two types of receptor are involved. Although it frequently

contributes little to delineation of the mechanism of drug action, such categorization provides a convenient basis for summarizing drug effects. A statement that a drug activates a specified type of receptor is a succinct summary of its spectrum of effects and of the agents that will antagonize it. Similarly, a statement that a drug blocks a certain type of receptor specifies the agents that it will antagonize and at what sites. However, it should be appreciated that the accuracy of this statement may be altered when additional receptor subtypes are identified or additional drug mechanisms or side effects are revealed.

Significance of Receptor Subtypes. As the diversity and selectivity of drugs have increased, it has become clear that multiple subtypes of receptors exist within many previously defined classes of receptors. Moreover, molecular cloning frequently has revealed the presence of several closely related subtypes of receptors where only a single species was thought to exist, and some receptor subtypes have been shown to be differentially expressed during development. Knowledge of receptor subtypes is of interest to the researcher and of utility to the clinician who desires to manipulate them.

In the case of the nicotinic cholinergic receptor, referred to above, there are distinct differences in the ligand-binding and functional properties between the receptors that are found in the ganglia of the autonomic nervous system and those at the somatic neuromuscular junction. This difference is exploited for therapeutic benefit. Thus, antagonists that act preferentially at the nicotinic receptors in ganglia can be used to control blood pressure without paralyzing skeletal muscle. Tubocurarine and related agents constitute the converse example; their ability to antagonize the action of acetylcholine is relatively well confined to the receptor sites at the neuromuscular junction (see Chapter 9). These subtypes of the nicotinic receptor as well as subtypes of, for example, β -adrenergic receptors (see Chapter 10) are conceptually analogous to tissue-specific isozymes of an enzyme.

Although the mechanisms of action of some receptor subtypes may be very similar, differing only slightly in kinetics or regulatory activity, other receptor subtypes display fundamental differences in their biochemical and cellular regulatory activities. Examples include the α_1 - and α_2 -adrenergic receptors and the M_1 - and M_2 -muscarinic cholinergic receptors. Although all four receptor subtypes regulate G proteins, the α_1 -adrenergic and the M_1 - (and M_3 -) muscarinic receptors initiate Ca^{2+} signaling via G_q , whereas the α_2 -adrenergic and the M_2 - (and M_4 -) muscarinic receptors regulate other signaling pathways via G_i and another GTP binding protein, G_o (see Chapters 7 and 10). Expression of such different receptor subtypes allows a single agonist to evoke unique responses in specific cells or tissues.

When a tissue or cell expresses more than a single subtype of receptor or when only insufficiently selective drugs are available, identification of the specific signal that is generated by an individual receptor requires more direct approaches. These include the expression of the cloned cDNA for the receptor in a well-characterized cell where its signaling activities can be studied in detail, the expression and purification of the recombinant receptor for direct biochemical

analysis of its functions, or the use of antisense strategies to evaluate which signal-transducing pathway is necessary for agonist effects.

The discovery by molecular cloning of numerous receptor subtypes raises the question of their importance, particularly when their signaling mechanisms and specificity for endogenous ligands are indistinguishable. Perhaps the multiplicity of genes facilitates their independent, cell-specific and developmentally controlled expression, allowing receptor subtypes to be expressed independently according to the developmental needs of the organism. Regardless of their mechanistic implications (or lack thereof), schemes of receptor classification have facilitated the development of a number of therapeutic agents that have selectivity for specific types or subtypes of receptors. Such drug development has provided the clinician with compounds having higher ratios of therapeutic effects to toxic or unwanted effects.

Actions of Drugs Not Mediated By Receptors

If one restricts the definition of receptors to macromolecules, then several drugs may be said not to act by virtue of combination with receptors. Certain drugs interact specifically with small molecules or ions that are normally or abnormally found in the body. One example is the therapeutic neutralization of gastric acid by a base (antacid). Another example is the use of mesna, a free radical scavenger rapidly eliminated by the kidneys, to bind to reactive metabolites associated with some cancer chemotherapeutic agents and thus minimize their untoward effects on the urinary tract (see Chapter 51). Other agents act more by virtue of their colligative effects than by more classical chemical mechanisms. Such mechanisms are characterized by a lack of requirement for highly specific chemical structure. For example, certain relatively benign compounds, such as mannitol, can be administered in quantities sufficient to increase the osmolality of various body fluids and thereby cause appropriate changes in the distribution of water (see Chapter 29). Depending on the agent and route of administration, this effect can be exploited to promote diuresis, catharsis, expansion of circulating volume in the vascular compartment, or reduction of cerebral edema.

Certain drugs that are structural analogs of normal biological chemicals may be incorporated into cellular components and thereby alter their function. This property has been termed a "counterfeit incorporation mechanism," and has been particularly useful with analogs of pyrimidines and purines that can be incorporated into nucleic acids;

such drugs have clinical utility in cancer and antiviral chemotherapy (see Chapters 50 and 51).

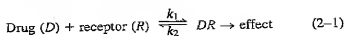
QUANTITATION OF DRUG-RECEPTOR INTERACTIONS AND ELICITED RESPONSE

As early as 1878, even before he coined the term *receptive substance*, Langley suggested that drug-cell combinations, and hence the actions and effects of drugs, were probably governed by the law of mass action. This view was developed further by A. J. Clark in the 1920s (see monograph published in 1933), and it remains basic to understanding the mechanisms of drug action.

Extending the analysis of drug-receptor interactions beyond the initial binding of drug to receptor raises important questions as to the relationship between the concentration of drug-receptor complex and the magnitude of the observed effect. In the classical receptor theory developed by Clark, it was assumed that the effect of a drug is proportional to the fraction of receptors occupied by drug and that maximal effect results when all receptors are occupied. While these assumptions may be true in limited cases, exceptions are common. Consequently, Ariens (1954) introduced the term *intrinsic activity* (or α , a "proportionality constant") to describe the relationship between the effect, E , elicited by a drug, D , and the concentration of drug-receptor complexes: $E = \alpha[DR]$. This relationship also addressed the anomalous observation that some drugs did not elicit a maximal response even at apparently maximal receptor occupancy. It was Stephenson (1956), however, who advanced the concept of concentration-response relationships even further by offering an explanation for nonlinear relationships between occupancy and response and for the ability of a group of agonists to produce equal responses while occupying different proportions of the receptor population. This explanation involves a property of an agonist that Stephenson called *efficacy*. In the terms of Stephenson, the response, R , of a tissue is some function of the stimulus, S , given to that tissue: $R = f(S)$ and $S = e\gamma$, where e = efficacy and γ = fractional receptor occupancy. Differences in drug effects in different tissues are thus a reflection of the contributions of properties of the drug, properties of its receptor, and properties of a tissue in terms of receptor density and the coupling of receptor occupancy to the ultimate response. Today, the terms *intrinsic activity* and *efficacy* are commonly used interchangeably and are operationally synonymous. Although the mathematical description of dose-response relationships cannot reveal molecular mechanisms, the basic descriptive concepts are instructive and provide an appreciation of how the concentration of a drug at its target organ determines the therapeutic response.

If one assumes that an agonist drug interacts reversibly with its receptor, that the resultant effect is proportional to the number of receptors occupied, and that a maximum effect occurs when all receptors are occupied, as in the original model of A. J. Clark, the following re-

action scheme can be written:



The relationship between effect and the concentration of free drug can be described simply for this model as:

$$\text{Effect} = \frac{\text{maximal effect} \cdot [D]}{K_D + [D]} \quad (2-2)$$

where $[D]$ is the concentration of free drug and K_D (equal to k_2/k_1) is the equilibrium dissociation constant for the drug-receptor complex. The fraction of receptors that is occupied by drug is equal to $[D]/(K_D + [D])$. This equation describes a simple rectangular hyperbola and is analogous to the Michaelis-Menten equation that is used to describe the interaction of enzyme and substrate where no product is formed (Figure 2-5, A). The scheme defines the drug's *potency*—that is, the dependency of effect on its concentration. It is frequently convenient to plot the magnitude of effect versus $\log [D]$, because a wide range of drug concentrations is easily displayed and the potency of different drugs can be compared readily. In this case, the result is the familiar sigmoidal log dose-effect curve, probably the most intuitively helpful graphical display of drug action (Figure 2-5, B). This representation also allows comparisons of the relative potencies and efficacies of agonists (Figure 2-5, C). When there is a linear relationship between occupancy and response, as in the model of A. J.

Clark, the concentration of the drug at which it is half-maximally effective, its EC_{50} , is equal to its K_D . However, as noted above, there often is amplification between occupancy and response, such that the EC_{50} for response lies far to the left of the K_D for receptor occupancy (Figure 2-5, D).

As stated earlier, *antagonists* bind to the receptor or components of the effector mechanism to inhibit the action of an agonist, but, in the classical definition, initiate no effect themselves. If the inhibition can be overcome by increasing the concentration of the agonist, ultimately achieving the same maximal effect, the antagonist is said to be *competitive* or *surmountable*. This type of inhibition commonly is observed with antagonists that bind reversibly at the receptor site. In the terminology of Stephenson, classical competitive antagonists would have zero efficacy. Because the maximal effect can still be achieved if sufficient agonist is used, the log dose-effect curve for the agonist is shifted to the right by a competitive antagonist (Figure 2-6, A). The maximal effect is unaltered, but the agonist appears to be less potent.

The parallel rightward shift in agonist concentration-response curves in the presence of increasing concentration of antagonist (Figure 2-6, B) provides an opportunity to further characterize antagonist properties in physiological preparations. Thus, calculation of the ratio of agonist concentrations that elicit equal responses in the absence and presence of antagonist at increasing concentrations (termed the dose ratio) and plotting these values according to the relationship $\log(\text{dose ratio} - 1)$ versus $\log[\text{antagonist}]$ yields, at the $y = 0$ intercept, the K_D value for antagonist at the receptor (Figure 2-6, C). This data transformation, referred to as the Schild regression, provides additional insight into the nature of the antagonist interaction with the receptor. Competitive antagonists interacting with a single

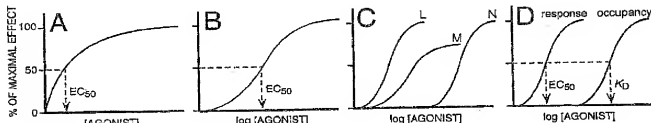


Figure 2-5. Agonist interactions with biological receptors.

A. In the simplest circumstance, agonist occupancy of the receptor obeys the law of mass action, and the relationship between agonist concentration (*linear scale*) and response is reflected by a rectangular hyperbolic relationship. B. A plot of response versus \log [agonist] reveals a sigmoidal relationship between occupancy and response, such that, in the absence of negative or positive cooperativity, 10% to 90% response occurs over approximately a 100-fold range of agonist concentration. C. Agonists vary in terms of *potency* and *efficacy*. The EC_{50} value represents the concentration of agonist that elicits a half-maximal response. Drug L is more potent than drugs M and N; drugs L and N are more efficacious than drug M, a *partial agonist*. D. Because occupancy often is not directly related to response and signal amplification occurs between receptor occupancy, effector activation, and ultimate response, dose-response curves often fall to the left of receptor-occupancy profiles.

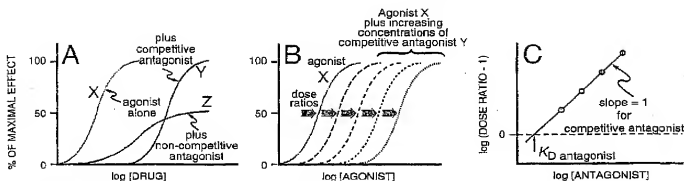


Figure 2-6. Properties of antagonist blockade of response.

A. Log dose-response curves for an agonist in the absence (X) or presence of a competitive (Y) or non-competitive (Z) antagonist. B. Agonist activation of a response in the presence of increasing concentrations of a competitive antagonist yields a series of parallel rightward shifted dose-response curves. The dose ratio is the ratio of the agonist concentrations (or doses) required to elicit equal responses in the presence and absence of antagonist. C. The Schild regression permits a direct estimate of the K_D for a competitive antagonist for receptor occupancy and hence blockade of response. When the slope of a Schild regression $\neq 1.0$, either the drug is not a competitive antagonist or experimental limitations to interpretation prevail, and the value of x at $y = 0$ has no thermodynamic significance (see Kenakin *et al.*, 1992).

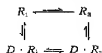
population of noninteracting receptors yield a regression line with a slope equal to 1. Antagonist interactions with multiple receptor subtypes having differing affinities for the antagonist or antagonist effects other than those that are strictly competitive and fully reversible yield complex Schild regression plots that are either nonlinear or diverge from a slope of 1 (Schild, 1957; Kenakin *et al.*, 1992). K_D values so derived should be the same when a single antagonist is used with several agonists that act on the same receptor.

A noncompetitive antagonist prevents the agonist, at any concentration, from producing a maximum effect on a given receptor. This could result from irreversible interaction of the antagonist at any site to prevent binding of agonist or from reversible or irreversible interaction with a component of the system so as to decrease or eliminate the effect of the binding of agonist. Intuitively, these results may be conceptualized as removal of receptor or the system's capacity to respond. The maximal effect possible is reduced, but agonist can act normally at receptor-effector units that are not influenced by antagonist. Typically, log dose-effect curves reflect reduced apparent efficacy but unaltered potency in the presence of a noncompetitive antagonist (Figure 2-6, A).

Antagonists may be classified as acting reversibly or irreversibly. If the antagonist binds at the active site for the agonist, reversible antagonists will be competitive, and irreversible antagonists will be noncompetitive. If binding is elsewhere, however, these simple rules do not hold, and any combination of functional outcomes is possible.

If two drugs bind to the same receptor at the same site, why can one be an agonist and the other produce no effect—acting as an antagonist? This central question of pharmacodynamics is also central to our understanding of protein structure and protein-ligand interactions. Its answer derives from two active and complementary experimental approaches. On the one hand, structural biophysicists have determined and compared the active and inactive structures of proteins whose activities are controlled by the binding of regulatory ligands. These studies have elucidated the molecular forces that allow one ligand but not another to alter a protein's conformation. Biochemical analysis of protein-ligand interactions, using both enzymatic activity and spectroscopic measures of conformational change, have produced a rich understanding of the energetics and kinetics of allosteric regulation that is consistent with the structural data (Wyman and Gill, 1973; Weber, 1994).

Consider that a receptor must, by definition, exist in at least two conformations: active (s) and inactive (i).



These conformations might correspond to the open and closed states of an ion channel, the active and inactive states of a protein tyrosine kinase, or the productive versus nonproductive conformations of a receptor for coupling to G proteins. The extent to which the equilibrium is perturbed is determined by the relative affinity of the drug for the two conformations (Figure 2-7). If these states are in equilibrium and the inactive state predominates in the absence of drug, then the basal signal output will be low. In this case, the presence of a drug that has a higher affinity for the active conformation than for the inactive conformation will drive the equilibrium to the active state

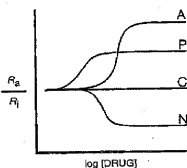


Figure 2-7. A working model for receptor-mediated response.

Effects of drugs on the relative concentrations of two hypothetical forms of a receptor, R_a (active) and R_i (inactive) that are in equilibrium, $R_a \rightleftharpoons R_i$. As discussed in the text, the relative distribution of the receptor between these two forms is differentially influenced by agonists (A), partial agonists (P), competitive antagonists (C), and negative antagonists (N), also known as inverse agonists.

and thereby activate the receptor. Such a drug will be an agonist. A full agonist is sufficiently selective for the active conformation so that, at a saturating concentration, it will drive the receptor completely to the active state (e.g., agent A in Figure 2-7). If a different but perhaps structurally analogous compound binds to the same site on R but with only slightly greater affinity for R_a than for R_i , the magnitude of effect observed may be less, even at saturating concentrations, such as agent P in Figure 2-7. A drug that displays such intermediate effectiveness is referred to as a *partial agonist*. Partial agonists are not hypothetical; they are common and were first described quantitatively in the formulations introduced by Ariens and Stephenson.

A drug that binds with equal affinity to either conformation will not alter the activation equilibrium and will act as a competitive antagonist, shown as drug C in Figure 2-6. A partial agonist also can act as an antagonist. When it binds to the receptor (and produces a submaximal response), it also occupies the drug binding site competitively with respect to a full agonist. A greater concentration of a full agonist will be required to produce a maximal effect because of this competition. A drug with preferential affinity for R_i will actually produce an effect opposite to that of an agonist, and examples of so-called *negative antagonists* or *inverse agonists* do exist (see Chapters 11 and 17). These agents have properties that are analogous to those of drug N in Figure 2-7. However, if the preexisting equilibrium lies far in the direction of R_i , negative antagonism may be difficult to observe and to distinguish from simple competitive antagonism. Careful biochemical studies of receptor-drug interactions, coupled with the analysis of receptors in which the intrinsic R_a/R_i equilibrium has been studied by mutation, have supported this model of drug action. Although the complete mathematical description can be complex (Taylor and Insel, 1990), the model is manageable and readily applicable to experimental data through the use of appropriate computer-assisted analysis. There is, then, the potential ability to design agents that suppress agonist-independent activity of receptors that are hyperactive in the so-called basal state, as opposed to agents that block receptor activity caused by excess availability of agonist.

As stated above, the terms *intrinsic activity* or *efficacy* are used to describe proportion of drugs that bind to the same receptor site but

do not produce equal maximum effects. Simplistically, the efficacy of a full agonist can be set equal to 1, that of an antagonist to 0, and that of a partial agonist to a value between 0 and 1. Fractional effect is then equal to the product of fractional occupancy of the receptor and the fractional efficacy. As first appreciated by Stephenson, these correction factors are dependent both on the molecular properties of receptors and on the concentrations and interactions of transducer and effector proteins. Thus, these factors are reliable only in a defined and controlled situation; they will change in different tissues and when different responses are measured. Nonetheless, these parameters are of use in evaluating and comparing the therapeutic utility of multiple drugs. Finally, it should be noted that the use of the word *efficacy* can, at times, be confusing. While a competitive antagonist has no efficacy in the sense that it does not serve as an initiator of an action that leads to a sequence of effects, it may have great therapeutic effectiveness, or *clinical efficacy*, when used to block agonist effects in human beings.

Even if the proximal molecular action of an agonist at a receptor is proportional to its efficacy and to the number of receptor sites occupied, the effects of subsequent steps in the signaling pathway frequently complicate the meaningful quantitative interpretation of the dose-dependent response. For example, while occupancy of a certain minimal number of receptors by an agonist may cause a proportional response, a later step in the pathway may become limiting at some greater level of stimulation. Further receptor occupancy then produces no additional effect. Thus, a plot of the drug's effect versus log concentration will lie to the left of a plot of fractional binding; drug potency will be greater than predicted by receptor affinity (Figure 2-5, D). This situation, in which a maximal apparent effect is achieved when a relatively small fraction of receptors is occupied, is explained by the concept of *spare receptors*. In at least some of these cases, a certain number of receptors can be lost (e.g., with an irreversible antagonist) without diminution of the maximal observable response. Note that the existence of spare receptors does not necessarily imply a molecular excess of receptors over effector or transducer proteins. Spare receptors frequently are encountered whenever a receptor acts catalytically rather than stoichiometrically. In the case of the receptors that are tyrosine protein kinases, a few agonist-occupied receptors may be sufficiently active to maintain the phosphorylation of a greater number of substrate protein molecules. Similarly, a single G protein-coupled receptor can maintain the activation of hundreds of G protein molecules in some settings.

Because cellular signal transducing pathways are designed to amplify and integrate a multiplicity of stimulatory and inhibitory signals, it should come as no surprise that the outcome of pharmacological intervention generally is a complex consequence of the proximal effect of a single drug at its receptor. Increasingly complex mathematical models can be derived to describe the phenomenologic be-

is. Simplistically, the effect of such systems, but analysis of the individual steps that of an antagonist at receptor-response pathway at a molecular level provides a more fruitful approach to identify new molecular targets for drug therapy.

PROSPECTUS

will change in different
assured. Nonetheless,
compating the therapies
uld be noted that the
fusing. While a competi
: that it does not serve a
equence of effects, it is
ical efficacy, when used

BIBLIOGRAPHY

- J. Drug Discovery on the assembly line. *Science*, 1994, 265:1399-1401.
1. its efficacy and action of agonist. In: *The Affinity and intrinsic activity in the theory of competitive inhibition*. I. Problems and theory. *Arch. Int. Pharmacodyn.*, 1954, 1, the effects of sub-agonists.
2. frequently complex. M.J. Inositol triphosphate and calcium signalling. *Nature*, 1994, 367:315-325.
3. occupancy of a certain. M. and Garbers, D.L. Signal transduction by guanylyl cyclases. *Rev. Biochem.*, 1991, 60:553-575.
4. an agonist may cause. D.E. Mutations in G protein-linked receptors: novel insights in the pathway. *Mol. Cell. Neurosci.*, 1993, 7:1237-1239.
5. el of stimulation. Fu, D.E., and Naei, E.J. New roles for G protein $\beta\gamma$ -dimers in transduction of stimulation. *Nature*, 1993, 365:403-406.
6. has no additional effect. *Neuroscience Signalling*, 1994, 1:1-3.
7. has log concentration of agonist. *The Mode of Action of Drugs on Cells*. E. Arnold and Co., 1935, 1:133.
8. binding; drug potency. A.M., Blumenthal, D.K., and Krebs, E.G. Protein serine receptor affinity (Figurine kinases). *Ann. Rev. Biochem.*, 1987, 56:567-613.
9. a maximal apparent K_m . The steroid and thyroid hormone receptor superfamily. *Small fraction of receptor*, 1988, 240:889-895.
10. the concept of spare receptors. J., Johnson, D. E., and Williams, L.T. Signaling by receptor tyrosine kinases. *Annu. Rev. Biochem.*, 1993, 62:453-481.
11. uses, a certain number of receptors. E., McPhail, M.J., Russell, D.W., and Wilton, J.D. The anorectic resistance syndromes. In: *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed. (Scriver, C.R., Beaudet, A.L., Sly, W.L., and Valle, D., eds.) McGraw-Hill, New York, 1995, 25:267-298.
12. receptors do not play a role. D., eds.) McGraw-Hill, New York, 1995, 25:267-298.
13. of receptors over the cell membrane. In: *An Introduction to Molecular Neurobiology*, 2nd ed. (Hall, ed.) Sinauer Associates, Inc., M.A., 1992, pp. 81-118.
14. receptors frequently play a role. B., Bond, R.A., and Bonner, T.I. Definition of pharmacological catalytically regulated receptors. *Pharmacol. Rev.*, 1992, 44:351-367.
15. of the receptors that are. Structure-based strategies for drug design and discovery. *Science*, 1992, 257:1078-1082.
16. agonist-occupied receptors. *Cell Surface Receptors: A Short Course on Theory and Experiment*, 2nd ed. M. Nijheff, Boston, 1995, 1:1-10.
17. are protein molecules. Landis, C.A., Farah, G., Vallar, L., Grunewald, K., Feichtinger, M., and Reuter, A.Y. Clark, O.H. Kawasaki, E., Bourne, H.R., and McRobert, M. Two G protein oncogenes in human endocrine tumors. *Science*, 1994, 264:555-559.
18. signal transducing pathways. J., D.J., Umesono, K., and Evans, R.M. The retinoid receptors. *Endocrine: Biology, Chemistry, and Medicine*, 2nd ed. (Sporn, M.B., and Roberts, A.B., and Goodman, D.S., eds.) Raven Press, New York, 1994, 1:1-10.
19. of the proximal effect of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
20. increasingly complex model. *Pharmacol. Rev.*, 1992, 44:351-367.
21. to describe the phenomena. *Pharmacol. Rev.*, 1992, 44:351-367.
22. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
23. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
24. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
25. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
26. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
27. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
28. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
29. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
30. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
31. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
32. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
33. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
34. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
35. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
36. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
37. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
38. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
39. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
40. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
41. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
42. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
43. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
44. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
45. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
46. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
47. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
48. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
49. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
50. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
51. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
52. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
53. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
54. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
55. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
56. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
57. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
58. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
59. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
60. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
61. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
62. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
63. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
64. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
65. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
66. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
67. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
68. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
69. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
70. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
71. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
72. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
73. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
74. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
75. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
76. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
77. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
78. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
79. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
80. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
81. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
82. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
83. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
84. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
85. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
86. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
87. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.
88. of the agonist. *Pharmacol. Rev.*, 1992, 44:351-367.

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT F

International Union of Pharmacology. XXIII. The Angiotensin II Receptors

M. DE GASPARO,¹ K. J. CATT, T. INAGAMI, J. W. WRIGHT, AND TH. UNGER

Novartis Pharma AG, Metabolic & Cardiovascular Diseases, Basel, Switzerland (M.d.G.); Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland (K.J.C.); Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tennessee (T.I.); Department of Psychology, Washington State University, Pullman, Washington (J.W.W.); Institute of Pharmacology, Christian-Albrechts-University of Kiel, Hospitalstrasse 4, Kiel, Germany (Th.U.)

This paper is available online at <http://www.pharmrev.org>

I. Introduction	417
A. Historical background	417
B. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification criteria for classification	418
C. Current nomenclature	419
D. Structural analysis	419
II. The type 1 (AT ₁) angiotensin receptor	420
A. Angiotensin II receptors: early studies	420
B. Cloned AT ₁ receptors	421
C. Genomic organization of rat AT _{1A} and AT _{1B} receptor genes	421
D. Expression and regulation of rat AT _{1A} and AT _{1B} receptor	422
E. The human AT ₁ receptor	422
1. AT ₁ receptor gene polymorphisms and cardiovascular disease	423
F. The amphibian AT ₁ receptor	423
G. The AT ₁ receptor null mouse	424
H. Structural basis of ligand binding to the AT ₁ receptor	425
1. Determinants of Ang II bioactivity	425
2. Agonist binding of the AT ₁ receptor	426
3. Antagonist binding of the AT ₁ receptor	427
I. AT ₁ receptor signaling mechanisms	430
1. AT ₁ receptor activation and signal transduction	430
2. AT ₁ receptor and tyrosine phosphorylation	432
3. AT ₁ receptor-activated growth responses	433
4. Transactivation of growth factor signaling by the AT ₁ receptor	434
5. Other AT ₁ receptor-mediated signaling pathways	434
J. Receptor activation and endocytosis	435
K. AT ₁ receptor function in selected tissues	436
1. The AT ₁ receptor and the brain	436
2. Ang II-induced neuronal signaling pathways	437
3. Role of Ang III in the brain	438
4. The AT ₁ receptor and the pituitary gland	438
5. The AT ₁ receptor and the heart	439
III. The type 2 (AT ₂) angiotensin receptor	440
A. Cloning, purification, and properties of the AT ₂ receptor	440
B. Regulation of the AT ₂ receptor	442
C. AT ₂ receptor diversity	442
D. Targeted AT ₂ receptor gene overexpression and deletion	443
1. Behavioral changes in AT ₂ receptor null mice	444
E. Signaling mechanisms of the AT ₂ receptor	444

¹ Address for correspondence: Marc de Gasparo, Novartis Pharma AG, Metabolic & Cardiovascular Diseases, WKL 121-210, P.O. Box 4200, Basel, Switzerland. E-mail: marc.de_gasparo@pharma.novartis.com and m.de_gasparo@bluewin.ch (after October 1, 2000)

1. Dephosphorylation and inactivation of the mitogen-activated protein kinases ERK1 and ERK2	445
2. Activation of phospholipase A ₂ and prostacyclin generation	446
F. Tissue distribution of the AT ₂ receptor	446
1. Brain	447
2. Heart	448
3. Kidney	449
4. Vasculature	450
5. Pancreas, lung, thymus, and other tissues	451
6. Cells in primary culture and cell lines expressing the AT ₂ receptor	451
G. Pathophysiological aspects of AT ₂ receptor activation	452
1. The AT ₂ receptor can induce apoptosis	452
2. Effects on vascular tone	453
3. Vascular hypertrophy and fibrosis and the AT ₂ receptor	453
4. Renal tubular function	453
5. Neuronal cell differentiation and nerve regeneration	454
H. Summary	454
IV. The AT ₄ receptor	455
A. Signaling mechanisms	455
B. Tissue distribution of the AT ₄ receptor	456
1. Brain	456
2. Peripheral tissue	456
C. Development of agonists and antagonists	456
1. Binding requirements of the AT ₄ receptor	456
2. Antagonists of the AT ₄ receptor	457
D. Physiology associated with the AT ₄ receptor	458
1. Regulation of blood flow	458
2. Cardiac hypertrophy	458
3. Renal tubular reabsorption	459
4. Electrophysiological analysis	459
5. Role of Ang IV in learning and memory	459
E. Summary	460
V. General conclusions	460
References	460

Abstract—The cardiovascular and other actions of angiotensin II (Ang II) are mediated by AT₁ and AT₂ receptors, which are seven transmembrane glycoproteins with 30% sequence similarity. Most species express a single autosomal AT₁ gene, but two related AT_{1A} and AT_{1B} receptor genes are expressed in rodents. AT₁ receptors are predominantly coupled to G_{q/11}, and signal through phospholipases A, C, D, inositol phosphates, calcium channels, and a variety of serine/threonine and tyrosine kinases. Many AT₁-induced growth responses are mediated by transactivation of growth factor receptors. The receptor binding sites for agonist and nonpeptide antagonist ligands have been defined. The latter compounds are as effective as angiotensin converting enzyme inhibitors in cardiovascular diseases but are better tolerated. The AT₂ receptor is expressed at high density during fetal development. It is much less abundant in adult tissues

and is up-regulated in pathological conditions. Its signaling pathways include serine and tyrosine phosphatases, phospholipase A₂, nitric oxide, and cyclic guanosine monophosphate. The AT₂ receptor counteracts several of the growth responses initiated by the AT₁ and growth factor receptors. The AT₄ receptor specifically binds Ang IV (Ang 3-8), and is located in brain and kidney. Its signaling mechanisms are unknown, but it influences local blood flow and is associated with cognitive processes and sensory and motor functions. Although AT₁ receptors mediate most of the known actions of Ang II, the AT₂ receptor contributes to the regulation of blood pressure and renal function. The development of specific nonpeptide receptor antagonists has led to major advances in the physiology, pharmacology, and therapy of the renin-angiotensin system.

I. Introduction

A. Historical Background

Blood pressure was measured for the first time in 1733 by Stephen Hales, in a dramatic experiment on a horse, by inserting a brass pipe into the carotid artery. The technique of modern blood pressure measurement was introduced in 1905 by Nicolai Korotkov using the stethoscope invented by Laennec in 1815 and the relatively recently devised wraparound inflatable rubber cuff. The latter was first described by Riva-Rocci in 1896 and was improved by von Recklinghausen in 1901 (Freis, 1995).

The first insight into the regulation of blood pressure came from the discovery of a pressor principle by Tigerstedt and Bergman in 1897. They called this factor "renin" because it was extracted from the kidney. This pioneering work led to the description of reno-vascular hypertension in animals and in humans (Goldblatt et al., 1934). However, it was not until 1940 (Braun-Menéndez et al., 1940) that a vasoconstrictor substance was isolated from renal venous blood from the ischemic kidney of a Goldblatt hypertensive dog. A similar finding was made simultaneously and independently by Page and Helmer (1940) after the injection of renin into an intact animal. This group also isolated a so-called "renin activator" that later proved to be angiotensinogen. The pressor substance was named "hypertensin" in Argentina and "angiotonin" in the United States and was later isolated and shown to be an octapeptide (Skeggs et al., 1956; Bumpus et al., 1957; Elliott and Peart, 1957). There were differences between laboratories concerning interpretations and nomenclature but in fact hypertension and angiotonin were the same substance. In 1958, Braun-Menéndez and Page agreed on the hybrid term angiotensin for the highly potent pressor octapeptide. This proved to be an appropriate choice, given the later recognition of angiotensin's numerous actions in addition to its hypertensive effects. The sequence of angiotensin II is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe in the human, horse, and pig. In bovine angiotensin II, the isoleucine residue in position 5 is replaced by valine.

Following this major discovery, the various components of the cascade leading to the formation of angiotensin II were characterized, including angiotensinogen, angiotensin converting enzyme (ACE),² and angio-

tensins I, II, and III (Table 1). The synthesis of the peptide angiotensin II by Bumpus et al. (1957) and by Rittel et al. (1957) was followed by a continuing series of investigations into the structure-activity relationship of angiotensin analogs, mainly in the hope of finding a peptide antagonist.

In 1987, a committee of the International Society for Hypertension, The American Heart Association, and the World Health Organization proposed abbreviating angiotensin to Ang using the decapeptide angiotensin I as the reference for numbering the amino acids of all angiotensin peptides (Dzau et al., 1987).

Angiotensin II plays a key role in the regulation of cardiovascular homeostasis. Acting on both the "content" and the "container", Ang II regulates blood volume and vascular resistance. The wide spectrum of Ang II target tissues includes the adrenals, kidney, brain, pituitary gland, vascular smooth muscle, and the sympathetic nervous system. Angiotensin is not only a blood-borne hormone that is produced and acts in the circulation but is also formed in many tissues such as brain, kidney, heart, and blood vessels. This has led to the suggestion that Ang II may also function as a paracrine and autocrine hormone, which induces cell growth and proliferation and controls extracellular matrix formation (Dzau and Gibbons, 1987; Griffin et al., 1991; Weber et al., 1995a,b). Other angiotensin-derived metabolites such as angiotensin 2-8 (Ang III), angiotensin 1-7, or angiotensin 3-8 (Ang IV) have all been shown to have biological activities (Table 1) (Peach, 1977; Schiavone et al., 1990; Ferrario et al., 1991; Ferrario and Iyer, 1998; Wright et al., 1995).

As for other peptide hormones, Ang II was postulated to act on a receptor located on the plasma membrane of its target cells. This receptor should possess the dual functions of specific recognition of the ligand and stimulation of the characteristic cellular response. Comparison of changes in steroidogenesis in the adrenal cortex, adrenal catecholamine release, and developed tension in aortic strips in response to Ang I, Ang II, and Ang III clearly indicated different affinities of these target organs for the three peptides (Peach, 1977; Devynck and Meyer, 1978). These pharmacological experiments showed that effector organs responded to Ang I, II, and

² Abbreviations: ACE, angiotensin converting enzyme; NC-IUPHAR, International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification; GPCR, G protein-coupled receptor; GTP- γ S, guanosine 5'- γ -O-(thio)triphosphate; PKC, protein kinase C; kb, kilobase(s); bp, base pair(s); RT-PCR, reverse transcriptase-polymerase chain reaction; IGF-1, insulin-like growth factor 1; NO, nitric oxide; NOS, NO synthase; VSMC, vascular smooth muscle cell(s); MAPK, mitogen-activated protein kinase; EPO, erythropoietin; CHO, Chinese hamster ovary; TMD, transmembrane domain; PK3, protein kinase B; EGF, epidermal growth factor; GAP, GTPase-activating protein; PDGF, platelet-derived growth factor; JNK, c-Jun N-terminal kinase; PAK, p21-activated

kinase; PLC, phospholipase; SFO, subfornical organ; OVLT, organum vasculosum lamina terminalis; AOTN, adrenocorticotropin; APA, aminopeptidase A; APN, aminopeptidase N; GnRH, gonadotropin-releasing hormone; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MKP-1, MAPK-phosphatase-1; PTP, phosphotyrosine phosphatase; PGE₂, prostaglandin E₂; IL, interleukin; IRS, insulin response sequence; IRF, interferon regulatory factor; NGF, nerve growth factor; NBC, Na⁺/HCO₃⁻ symporter system; NHE, Na⁺/H⁺ exchanger; PAI, plasminogen activator inhibitor; Ang, angiotensin; L-NAME, N^G-nitro-L-arginine; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; TMD, transmembrane domain; JAK, Janus cytosolic protein kinase; STAT, signal transducers and activators of transcription.

TABLE 1
Amino acid sequences of Ang II precursors and metabolites

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Angiotensinogen	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu				
Ang I	Leu	Val	Tyr	His										
Ang II	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu				
Ang III	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe						
Ang IV	Arg	Val	Tyr	Ile	His	Pro	Phe							
Angiotensin 1-7	Val	Tyr	Ile	His	Pro	Phe								
	Asp	Arg	Val	Tyr	Ile	His	Pro							

III with 2 to 3 log differences in potency from tissue to tissue. Based on these studies, Ang II receptor selectivity for the agonists was proposed to be structure-activity related. Comparison of Ang II and a large number of synthetic agonists and antagonists formed by substituting various amino acids of Ang II indicated marked dissimilarities between the analogs in each of the preparations, suggesting differences in the structure of the receptor sites (Khosla et al., 1974; Papadimitriou and Worcel, 1974; Peach and Levens, 1980).

Early binding studies detected sites with binding characteristics that differed between the various target tissues (Peach and Levens, 1980). Also, receptor density was up- or down-regulated in different tissues following either Ang II infusion or Na⁺ restriction (Aguilera and Catt, 1978). The characterization of receptor types in rat liver and kidney cortex (Gunther, 1984; Douglas, 1987; Bouscarel et al., 1988b) suggested further Ang II receptor heterogeneity. An early classification proposed for Ang II receptor types was based on studies in only a few tissues or species (Levens et al., 1980; Peach and Levens, 1980; Ferrario et al., 1991). It was not until the end of the 1980s that tools became available to demonstrate the existence of at least two receptor types in many tissues for which the conventional peptide analogs such as saralasin have high affinity but little or no selectivity. These included the nonpeptide antagonists losartan (or Ex89 or DuP 753) and PD123177, and a new generation of peptide ligands such as CGP42112 and α -aminophenylalanine Ang II (Chiu et al., 1989a; Whitebread et al., 1989; Speth and Kim, 1990). This new development was made simultaneously and independently in three different laboratories, and the initial nomenclature was confusing: the receptor sensitive to losartan was called 1, B, or α , and that with no affinity for losartan was termed 2, A, or β . The High Blood Pressure Research Council in 1990 and the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) in 1992 therefore appointed a subcommittee³ to address the problem, and a classification was proposed in 1991 and updated in 1995 (Bumpus et al., 1991; de Gasparo et al., 1995).

B. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification Criteria for Classification

To obtain a "fingerprint" capable of identifying distinct receptors, three main criteria have been proposed: operational, transductional, and structural (Humphrey et al., 1994). The operational criteria include the drug-related characteristics of the receptor, such as ligand binding affinities, and selective agonists and antagonists. The receptor-effector coupling events constitute the transductional criteria, and the receptor sequence and gene cloning represent the structural criteria. It is clear that all of these criteria are not necessarily achieved simultaneously and at an early stage. The coupling mechanism may not have a major influence on receptor pharmacology but it helps in differentiating receptor types. Also, receptors with diverse structures may respond to the same endogenous ligands. Finally, receptors may be cloned without having a known pharmacology. The combination of the three criteria should clearly help in defining true receptor types.

Any such classification will essentially evolve as our knowledge increases. Nevertheless, there is a need for an official scheme that will help to avoid confusion among investigators. Two Ang II receptor types fulfill the three classification criteria, and are termed AT₁ and AT₂ receptors. According to the NC-IUPHAR recommendation, the AT₁ and AT₂ receptors have an IUPHAR Receptor Code of 2.1.Ang.01.000.00.00 and 2.1.Ang.02.000.00.00 (Humphrey and Barnard, 1998). The first two numbers indicated the structural class: they are seven transmembrane domain, G protein-coupled receptor (GPCR) member of the rhodopsin subclass (2.1). The receptor family is abbreviated Ang. The types indicated as 01 and 02 for AT₁ and AT₂. The following series of null are reserved for splice variants chronologically numbered according to identification within species.

Two other receptors (AT₃ and AT₄) have been proposed, based on operational criteria, but their transduction mechanisms are unknown and they have not yet been cloned. The name AT₃ was initially given to a binding site described in the Neuro-2a mouse neuroblastoma cell line that was not blocked by either AT₁-specific losartan, or AT₂-specific PD123179 and was not affected by GTP analogs (Chaki and Inagami, 1992). This AT₃ binding site, which has a low affinity for Ang III, should

³ Members of the NC-IUPHAR Subcommittee on Angiotensin Receptors: R. Wayne Alexander, Kenneth E. Bernstein, Andrew T. Chiu, Theodore Goodfriend, Joseph W. Harding, Ahsan Hussain, Pieter B. M. W. Timmermans.

be called a non-AT₁-non-AT₂ site until more information about its nature has been obtained. The endogenous ligand for the AT₄ receptor is Ang 3-8 or Ang IV. Its binding properties and physiological characteristics, described in more detail in another section, are sufficiently different from those of the AT₁ and AT₂ receptor to warrant keeping the name AT₄ for this putative Ang IV-selective receptor until the binding protein is cloned and further characterized.

C. Current Nomenclature

The present angiotensin receptor identification is based on six principles. 1) The receptor is abbreviated to AT followed by a numerical subscript. 2) Further subdivisions are indicated by subscript letters that are in upper case for pharmacologically defined receptor subtypes (e.g., AT_{1B}). 3) The species is identified by a lowercase prefix preceding AT (e.g., r AT₁, h AT₂). There is a space between the species and the receptor name. 4) Mutant receptors should be designated with specification of the position of the amino acid substitution in bracket (e.g., [L112P]AT_{1A} when leucine at position 112 has been changed to proline. 5) The human gene is written in upper case and preferably but not essentially in italics (e.g., *AGTR1* and *AGTR2*). In mouse and rats, it would be *Agtr1a*, *Agtr1b* and *Agtr2* in lowercase.

D. Structural Analysis

The strategy of expression cloning was successfully applied to the AT₁ receptors of rat smooth muscle and bovine adrenal gland, and subsequently the corresponding receptors of mouse, rabbit, human, pig, dog, turkey, and frog angiotensin receptors were cloned and sequenced. The nonmammalian receptors have 60% identity with the mammalian receptor and are pharmacologically distinct in their ligand binding properties. Hydropathy analysis indicated that both AT₁ and AT₂ receptors contain seven hydrophobic transmembrane segments forming α helices in the lipid bilayer of the cell membrane. The structural information for the AT₁ receptor is coded as follows: h 359 aa, P30556, chr.3. This indicates that the human AT₁ receptor contains 359 amino acids, with the sequence reported in the SwissProt file under the number 30556 and the gene coding for the receptor (abbreviated *AGTR1*) is located on chromosome 3 q. Similarly, the structural coding for rat and mouse AT₁ receptor is r 359 aa, P29089, P25095 and m 359 aa, P29754, P29755 as there are two subtypes A and B in rat and mouse located on chromosomes 17 and 2 and 13 and 3, respectively. The AT₂ receptor is only 34% identical with its AT₁ counterpart (Fig. 1). The structural information is coded h 363 aa, P50052, chr.X

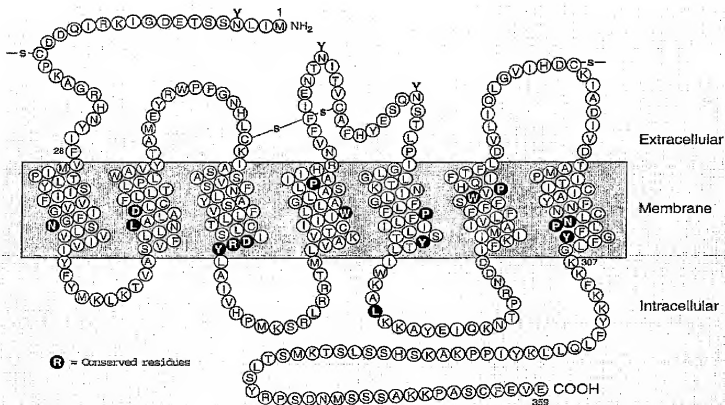


Fig. 1. Secondary structure and consensus sequence of the mammalian angiotensin AT₁ receptor. The amino acid sequence shown is based on the derived sequences of five individual cloned mammalian AT₁ receptors. The amino acid residues that are highly conserved among G protein-coupled receptors are indicated by bold letters. The positions of the three extracellular carbohydrate chains, and of the two extracellular disulfide bonds, are also indicated.

q22-q23 as the gene *AGTR2* is located on human chromosome X with the cytogenetic location q23-q24. For rat and mouse, the respective information is r 363 aa, P35361 and m 363 aa, P35374. As in human, the AT_2 receptor in rodents is also located on chromosome X.

An evolutionary analysis based on the alignment of cloned AT_1 receptor sequences, using the CLUSTAL algorithm of PC/gene, has suggested that rat and mouse AT_1 receptors coevolved. (Sandberg, 1994). Amphibian and avian receptors diverged early during evolution. So far, gene duplication has been observed only in rats and mice (see following section). Two isoforms of the AT_1 receptor derived by alternative splicing of the same gene have been reported in man (Curnow et al., 1995). They have similar binding and functional properties. A receptor with as much as 97% identity to the AT_1 receptor has been cloned from human placenta (Konishi et al., 1994). It differs in its C-terminal amino acid sequence, tissue distribution, and pharmacological properties. The gene has not been cloned and it may well be a splice variant of the AT_1 receptor.

II. The Type 1 (AT_1) Angiotensin Receptor

The angiotensin AT_1 receptor mediates virtually all of the known physiological actions of angiotensin II (Ang II) in cardiovascular, renal, neuronal, endocrine, hepatic, and other target cells. These actions include the regulation of arterial blood pressure, electrolyte and water balance, thirst, hormone secretion, and renal function. The AT_1 receptor belongs to the G protein-coupled receptor (GPCR) superfamily and is primarily coupled through pertussis toxin-insensitive G proteins to the activation of phospholipase C and calcium signaling. The AT_1 receptors of several species have been cloned and their amino acid sequences determined from the respective cDNAs. Ang II binding to the AT_1 receptor induces a conformational change in the receptor molecule that promotes its interaction with the G protein(s), which in turn mediate signal transduction via several plasma membrane effector systems. These include enzymes, such as phospholipase C, phospholipase D, phospholipase A_2 , and adenylyl cyclase, and ion channels, such as L-type and T-type voltage-sensitive calcium channels. In addition to activating several intracellular signaling pathways that mediate agonist-induced phenotypic responses in a wide variety of Ang II target cells, the agonist-occupied AT_1 receptor undergoes desensitization and internalization in the same manner as many other GPCRs.

The cellular responses to AT_1 receptor signaling include smooth muscle contraction, adrenal steroidogenesis and aldosterone secretion, neuronal activation, neurosecretion, ion transport, and cell growth and proliferation. The AT_1 receptor is coupled not only to the well recognized G_q -mediated calcium and protein kinase C signaling pathways, but also to intracellular signaling

casades that extend into the nucleus. These pathways regulate gene transcription and the expression of proteins that control growth responses and cell proliferation in several Ang II target tissues. Some of the latter consequences of AT_1 receptor activation are counteracted by the structurally dissimilar AT_2 receptor, which antagonizes the effects of AT_1 -mediated growth responses in several cell types, in particular endothelial cells, cardiomyocytes, and ovarian granulosa cells. These actions of the AT_2 receptor are described in more detail below. This account of the AT_1 receptor will address its gene expression, ligand binding, activation and signal transduction pathways, and physiological roles in the regulation of the activity and growth of its major target cells in cardiovascular, neuronal, and endocrine tissues.

A. Angiotensin II Receptors: Early Studies

The angiotensin receptor was identified as a functional entity by Lin and Goodfriend (1970), who first described the binding of radiolabeled Ang II to its receptor sites in the adrenal gland. These sites were subsequently shown to be located in the plasma membrane (Glossmann et al., 1974a), and the binding reaction was found to be influenced by the ambient Na^+ concentration and guanyl nucleotides (Glossmann et al., 1974b,c). The G proteins had not been discovered at that time, but this finding indicated that the binding activity of a noncyclic AMP-coupled receptor is regulated by guanine nucleotides. Subsequent studies showed that the AT_1 receptor is coupled to both G_q and G_i proteins in the adrenal glomerulosa zone and several other tissues in the rat.

Many of the properties of the angiotensin II receptor were first identified in studies on the adrenal gland and liver, both of which are abundant sources of receptors that are coupled to well defined physiological responses (Saltman et al., 1975; Campanile et al., 1982). As in the rat adrenal gland, guanine nucleotides reduced agonist binding of [^{125}I]-Ang II to hepatic receptors, largely by increasing its dissociation rate constant. Guanine nucleotides also decreased the number of high-affinity binding sites for Ang II, but not those for the peptide antagonist, [^{3}H]-Sar¹,Ala⁸]-Ang II. These changes were accompanied by inhibition of adenylyl cyclase activity in hepatic membranes, and of cyclic AMP production in intact hepatocytes (Crane et al., 1982). The high-affinity Ang II receptors in the liver were found to be inactivated by dithiothreitol, with a concomitant loss of Ang II-induced stimulation of glycogen phosphorylase in isolated hepatocytes (Gunther, 1984). These and related studies also presaged the existence of angiotensin II receptor types with distinct biochemical properties and intracellular mechanisms of action. Differential effects of guanine nucleotides on receptor binding of Ang II agonist and antagonist ligands were also observed in the bovine adrenal gland. This effect was evident for both membrane-bound and solubilized receptors. Concerning the latter, the association of the agonist-occu-

pled receptor with a putative G protein was suggested by its larger size on steric exclusion HPLC (De Lean et al., 1984).

The ability of Ang II to inhibit glucagon-stimulated cyclic AMP production in hepatocytes, and adenylyl cyclase activity in hepatic membranes, was consistent with its coupling to an inhibitory G protein, now termed G_i . This was confirmed by the ability of pertussis toxin to prevent the inhibitory action of Ang II on adenylyl cyclase. The ability of GTP γ S to further reduce receptor binding affinity when all G_i molecules were ADP-ribosylated by the toxin indicated that Ang II receptors are also coupled to other G protein(s) that could mediate actions of Ang II on additional signaling pathways (Pobiner et al., 1985). Subsequent studies on cultured hepatocytes revealed a single population of Ang II binding sites and demonstrated that agonist and antagonist analogs had parallel actions on cytosolic calcium and phospholipase activity, as did treatment with dithiothreitol to inactivate the receptors (Bouscarel et al., 1988a). Reconstitution studies in hepatocyte membranes showed that G_{i2} is the major form of G_i in these cells and is responsible for coupling the Ang II receptor to agonist-induced inhibition of adenylyl cyclase (Pobiner et al., 1991). One of the few physiological actions of Ang II that is mediated by G_i , rather than $G_{q/11}$, is the AT_1 receptor-dependent stimulation of angiotensinogen production in the rat liver (Klett et al., 1993).

B. Cloned AT_1 Receptors

The relatively low abundance of the AT_1 receptor in most Ang II target tissues, and the instability of the solubilized receptor molecule, impeded efforts to isolate and sequence the receptor protein. For this reason, expression cloning from bovine adrenal and rat smooth muscle cells was necessary to isolate the cDNAs encoding the receptor proteins of these species (Sasaki et al., 1991; Murphy et al., 1991). Both AT_1 receptors were found to be typical seven transmembrane domain proteins, composed of 359 amino acids and with a molecular mass of about 41 kDa. The extracellular regions, composed of the N terminus and the three extracellular loops, contain three N-glycosylation sites and four cysteine residues (Fig. 1). Each of the consensus sites is glycosylated in the native AT_1 receptor (Jayadev et al., 1999), which has a molecular mass of about 65 kDa. In addition to the two conserved cysteines that form a disulfide bond between the first and second extracellular loops of all GPCRs, the AT_1 receptor contains an additional pair of extracellular cysteine residues. These are located in the N-terminal region and the third extracellular loop, and form a second disulfide bond that maintains the conformation of the AT_1 receptor protein (Ohshima et al., 1995). The latter disulfide bond, which is not present in the AT_2 receptor, renders the AT_1 receptor susceptible to inactivation by dithiothreitol and other reducing agents. The cytoplasmic region of the

receptor, which is composed of the three intracellular loops and the C-terminal cytoplasmic tail, contains consensus sites for phosphorylation by several serine/threonine kinases, including protein kinase C (PKC) and GPCR kinases. Several of the specific residues that are phosphorylated during AT_1 receptor activation have been identified, but there are no confirmed reports of agonist-induced tyrosine phosphorylation of the AT_1 receptor or other GPCRs.

Similar structural features are present in several other cloned mammalian and nonmammalian AT receptors. The rat and mouse AT_1 receptors exist as two distinct subtypes, termed AT_{1A} and AT_{1B} , that are 95% identical in their amino acid sequences. The two subtypes are also similar in terms of their ligand binding and activation properties but differ in their tissue distribution, chromosomal localization, genomic structure, and transcriptional regulation. None of the other cloned mammalian AT_1 receptors, including those from cow (Sasaki et al., 1991), human (Bergsma et al., 1992; Curnow et al., 1992), pig (Itazaki et al., 1993), rabbit (Burns et al., 1993), and dog (Burns et al., 1994) appear to have subtypes. The two AT_1 subtypes in the rodent genome may be the consequence of a gene duplication event that occurred during evolution after the branching of rodents from the mammalian phylogenetic tree (Aiyar et al., 1994b).

C. Genomic Organization of Rat AT_{1A} and AT_{1B} Receptor Genes

The rat AT_{1A} receptor gene is 84 kb in length and contains three introns and four exons, the third of which (~2 kb) includes the entire 1077-base pair (bp) coding sequence of the receptor protein as well as 5' and 3' untranslated sequences (Langford et al., 1992; Murasawa et al., 1993; Takeuchi et al., 1993). The first two small exons encode alternatively spliced 5' untranslated sequences, and the fourth exon (1 kb) encodes an additional 3' untranslated sequence. A 2.3-kb transcript is found in all AT_{1A} -expressing tissues and contains exons 2 and 3. An additional 3.3-kb transcript containing exons 2, 3, and 4 is present in vascular smooth muscle cells and several other tissues but is not found in the brain. The transcription start site of the AT_{1A} receptor gene is located about 70 kb upstream from the exon that encodes the receptor protein. The rat AT_{1B} receptor gene is about 15 kb in length and contains two introns and three exons, the first two of which encode 5' untranslated sequences. The third exon contains the entire coding region of the receptor and the 3' untranslated sequence. The AT_{1B} receptor has 92 and 95% homology with the AT_{1A} at the nucleotide level and amino acid levels, respectively (Guo and Inagami, 1994) and is expressed in relatively few tissues as a 2.4-kb transcript. The rat AT_{1A} and AT_{1B} receptor genes are located on chromosomes 17q12 and 2q24, respectively (Tissir et al., 1995).

D. Expression and Regulation of Rat AT_{1A} and AT_{1B} Receptor

AT_{1A} and AT_{1B} receptors exhibit similar ligand binding and signal transduction properties but differ in their tissue distribution and transcriptional regulation. In the rat, AT_{1A} and AT_{1B} receptor mRNAs are expressed in numerous tissues, including adrenal, kidney, heart, aorta, lung, liver, testis, pituitary gland, and brain. AT_{1A} transcripts are predominantly expressed in all tissues except the adrenal and pituitary glands, where the AT_{1B} message is the major subtype. AT_{1A} receptors are abundantly expressed in vascular smooth muscle cells, in which their properties and regulation have been extensively investigated. In the adult mouse, AT_{1A} receptors are expressed in the kidney, liver, adrenal gland, ovary, brain, testis, lung, heart, and adipose tissue. In contrast, AT_{1B} receptors are confined to the adrenal gland, brain, and testis (Burson et al., 1994).

Studies on the tissue-specific expression of AT₁ receptor by *in situ* hybridization revealed that liver, heart, and lung contain solely AT_{1A} receptors, whereas the anterior pituitary gland contains only AT_{1B} receptors (Gasc et al., 1994). In the adrenal gland, the zona glomerulosa contains both AT_{1A} and AT_{1B} transcripts, the zona fasciculata contains little of either subtype, and only AT_{1A} mRNA is present in the medulla. In the kidney, AT_{1A} mRNA is present in mesangial and juxtaglomerular cells, proximal tubules, vasa recta, and interstitial cells, whereas AT_{1B} mRNA is found only in mesangial and juxtaglomerular cells, and in the renal pelvis. In male rats, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) showed that the relative abundance of AT_{1A} transcripts is 100% in liver, 85% in lung, 73% in kidney, 48% in adrenals, and 15% in the pituitary gland (Llorens-Cortes et al., 1994). In contrast to the adult animal, only AT_{1A} receptors are expressed in the pituitary gland during fetal and postnatal life.

The expression of the AT_{1A} receptor is stimulated by glucocorticoids, which act via one of three putative glucocorticoid responsive elements located in its promoter region (Guo et al., 1995). In the rat heart, where the AT_{1A} receptor is expressed in 10-fold excess over the AT_{1B} receptor, treatment with dexamethasone increased AT_{1A} and AT_{1B} mRNA levels by 100 and 300%, respectively (Della Bruna et al., 1995). Conversely, deoxycorticosterone acetate suppressed AT_{1A} mRNA levels by 70%, indicating that glucocorticoids and mineralocorticoids exert reciprocal actions on AT_{1A} receptor levels in the heart. In the heart and aorta, transcripts for both AT₁ subtypes were reduced by treatment with an AT₁ receptor antagonist. However, the AT_{1B} subtype was preferentially reduced, suggesting that the expression of AT_{1B} receptors in the adrenal is dependent on the activity of the renin-angiotensin system (Kitami et al., 1992).

Estrogens also influence the expression of AT₁ receptors, and exert divergent actions on subtype abundance in the pituitary gland and vascular smooth muscle. Estrogen treatment suppresses the expression of AT_{1B} but not AT_{1A} mRNA in the pituitary gland (Kakar et al., 1992). On the other hand, AT_{1A} receptor expression in vascular smooth muscle is elevated in ovariectomized rats and restored to normal by estrogen replacement (Nickenig et al., 1996). In cultured vascular smooth muscle cells, a high concentration of estradiol (1 μ M) reduced AT_{1A} mRNA by about 30%. Whether estrogen deficiency leads to increased vascular AT₁ receptor expression in the human has yet to be determined.

Other forms of hormonal regulation of AT₁ receptor expression include the insulin-induced up-regulation of vascular AT₁ receptor expression, which has been attributed to a post-translational mechanism (Nickenig et al., 1998). In cultured vascular smooth muscle cells, insulin caused a doubling of AT₁ receptor density and a concomitant increase in the Ang II-induced intracellular Ca²⁺ response. This increase in receptor content, which was dependent on tyrosine phosphorylation and the intracellular Ca²⁺ response, was due to an increase in receptor mRNA stability rather than increased gene transcription. In rat astrocytes, growth hormone but not insulin-like growth factor 1 (IGF-1) also increased AT_{1A} receptor expression. This was associated with an increase in gene transcription and elevated mRNA levels. AT_{1B} receptors, which were much less abundant than the AT_{1A} subtype, were not affected by growth hormone treatment (Wyse and Sernia, 1997). On the other hand, nitric oxide (NO) caused a marked decrease in AT_{1A} gene expression in vascular smooth muscle cell (VSMC) that was independent of changes in cyclic GMP. This was accompanied by an inhibitory action of NO on the expression of a reporter gene containing 616 bp of the AT₁ receptor gene promoter, and reduced association with a DNA binding protein that interacts with this region (Ichiiki et al., 1998).

E. The Human AT₁ Receptor

The human AT₁ receptor contains 359 amino acids, and its deduced amino acid sequence is 95% identical with those of the rat and bovine AT₁ receptors (Curnow et al., 1992; Bergsma et al., 1992; Furuta et al., 1992). The receptor is derived from a single large gene that contains five exons ranging in size from 59 to 2014 bp (Guo et al., 1994). The open reading frame of the AT₁ receptor is located on exon 5. The other four exons participate to varying degrees in alternative splicing to produce mature RNAs that encode two receptor isoforms that are translated with different efficiencies (Curnow et al., 1995). The inclusion of exon 2 occurs in up to 50% of AT₁ mRNAs and inhibits the translation of the downstream AT₁ receptor sequence. In about one-third of AT₁ transcripts, the splicing of exon 3 to exon 5 yields a receptor with a 32 amino acid N-terminal extension. The

ligand binding and signaling properties of this receptor are similar to those of the predominant shorter isoform of the AT₁ receptor.

The human AT₁ receptor gene is located on the q22 band of chromosome 3 (MEM number 106165) (Curnow et al., 1992; Davies et al., 1994). An additional human AT₁ receptor gene was suggested by the report of a human cDNA clone that differed from the known sequence in 10 of its 359 residues (Konishi et al., 1994), but subsequent studies have not confirmed the existence of a second gene (Curnow, 1996; Su et al., 1996). However, most human Ang II target tissues also express the slightly longer and functionally similar AT₂ receptor that results from alternative splicing of exons 3/5 as noted above. The longer isoform appears to be better expressed at the plasma membrane in cell transfection studies, but there is no evidence to suggest that it has a significant physiological role in AT₁ receptor function (Curnow, 1995).

Expression of the human AT₁ receptor is enhanced by epidermal growth factor in transfected COS-7 cells (Guo and Inagami, 1994b). Relatively little is known about the control of expression of the AT₁ receptor in most Ang II target tissues in the human. In the reproductive system, both Ang II and its AT₁ and AT₂ receptor types are present in the endometrium and exhibit cyclic changes during the menstrual cycle with a maximum in the early secretory phase (Ahmed et al., 1995). AT₁ receptors are expressed in the glands and the endometrial blood vessels and may participate in uterine vascular regulation and regeneration of the endometrium after menstruation. The human placenta expresses the AT₁ receptor and all other components of the renin-angiotensin system. The receptors are present throughout gestation in the syncytiotrophoblast and cytotrophoblast, and in the fetal vascular endothelial cells (Cooper et al., 1999). AT₁ receptor mRNA transcripts (2.4 kb) and receptor protein (83 kDa) increase progressively during pregnancy and reach their maximal level in the term placenta (Petit et al., 1996).

A local renin-angiotensin system is also present in human adipose tissue, with expression of angiotensinogen, ACE, and AT₁ receptor genes in omental and s.c. fat and cultured adipocytes (Engeli et al., 1999). The extent to which these components are related to the development of hypertension and obesity-related disorders has yet to be established. In the human kidney, AT₁ receptors are expressed in the renal vasculature, glomeruli, and the vasa recta bundles in the inner stripe of the outer medulla (Goldfarb et al., 1994). AT₁ receptors are diminished in the glomeruli of patients with chronic renal disease (Wagner et al., 1999). The AT₁ receptors expressed in cultured human mesangial cells mediate Ang II-induced hypertrophy and proliferative responses, implying that Ang II may be involved in the pathogenesis of glomerulosclerosis (Orth et al., 1995).

Similar effects of Ang II are mediated by AT₂ receptors in human pulmonary artery smooth muscle cells, in which Ang II stimulates DNA and protein synthesis. This response was associated with activation of mitogen-activated protein kinase (MAPK) and was prevented by losartan and by the MAPK inhibitor, PD-98059. These findings suggest that Ang II-induced activation of the AT₂ receptor initiates signaling pathways that participate in growth and remodeling of the human vascular system (Morrell et al., 1999).

In erythroid progenitor cells, which express both AT₁ and erythropoietin (EPO) receptors, Ang II enhances EPO-stimulated erythroid proliferation in vitro (Mrug et al., 1997). In vivo, the β_2 -adrenergic receptor-induced production of EPO in normal subjects was inhibited by losartan treatment, implying that Ang II is a physiological regulator of EPO production in the human (Freundenthaler et al., 1999).

1. AT₁ Receptor Gene Polymorphisms and Cardiovascular Disease. The discovery of several polymorphisms in the human AT₁ receptor gene, one of which (A1166C) was more frequent in hypertensive subjects (Bonardeaux et al., 1994), initiated a series of studies on the role of such mutations in the genesis of hypertension and other cardiovascular disorders. Subsequently, this polymorphism was reported to act synergistically with the angiotensin converting enzyme DD genotype on the risk of myocardial infarction (Tiret et al., 1994). However, the results of subsequent reports on this topic have not been consistent. In some studies, the A1166C polymorphism had no effect on ambulatory blood pressure, left ventricular mass, or carotid arterial wall thickness (Castellano et al., 1996; Schmidt et al., 1997). In other reports, the same AT₁ receptor gene polymorphism was associated with increased coronary arterial vasoconstriction in response to methylergonovine maleate (Amant et al., 1997), essential hypertension (Szombathy et al., 1998; Kainulainen et al., 1999), and increased left ventricular mass but not hypertension (Takami et al., 1998). An analysis of the role of this polymorphism in rats overexpressing the mutant human AT₁ receptor in the myocardium suggested that it is associated with increased responsiveness to Ang II. This may lead to cardiac hypertrophy under high-renin conditions or during pressure and volume overload (Van Geel et al., 1998).

F. The Amphibian AT₁ Receptor

In the *Xenopus laevis* oocyte, endogenous Ang II receptors were detected in the ovarian follicular cells that surround the oocyte. These receptors mediate Ang II-induced elevations of cytoplasmic Ca²⁺ in the oocyte via gap junctions between follicular cells and oocyte (Sandberg et al., 1990, 1992b) and are thus functionally identifiable as AT₁ receptors. However, the amphibian (xAT₁) receptor for Ang II did not recognize the nonpeptide antagonist, DuP753, that inhibits the binding and ac-

tions of Ang II at the mammalian AT_1 receptor (Sandberg et al., 1991). The xAT receptor cDNA was cloned from a *Xenopus* myocardial cDNA library to investigate the structural basis of this functional distinction in ligand binding. The xAT receptor is a 41-kDa protein containing 362 amino acids that has 60% amino acid identity and 65% nucleotide homology with the coding regions of known mammalian AT_1 receptors (Ji et al., 1993; Aiyar et al., 1994a). When expressed in *Xenopus* oocytes, xAT receptors mediate Ang II-induced Ca^{2+} mobilization and are pharmacologically distinct from mammalian AT_1 receptors. Receptor transcripts are present in *Xenopus* lung, liver, kidney, spleen, and heart, but not in adrenal, intestine, and smooth muscle. Mutational analyses of xAT and rat AT_1 receptors have largely elucidated the structural basis of their individual ligand binding properties, as described below.

G. The AT_1 Receptor Null Mouse

Gene targeting experiments have provided several important insights into the physiological role of the renin-angiotensin system in cardiovascular regulation, fluid and electrolyte balance, and development. Deletion of the genes encoding angiotensinogen (Tanimoto et al., 1994; Kim et al., 1996) and ACE (Krege et al., 1995; Esther et al., 1996) revealed that the lack of Ang II in *Agt*^{-/-} or *Ace*^{-/-} mice was associated with hypotension, reduced survival, and marked abnormalities in renal development. Most of the *Agt* null animals died before weaning and most of the *Ace* null mice died within 12 months. The *Agt* and *Ace* null animals that survived until adult life had severe renal lesions. In both cases, the kidneys showed focal areas of cortical inflammation, thickened arterial walls, and medullary hypoplasia with a consequent deficit in urinary concentration. An additional feature of interest in the *Ace*^{-/-} animals was impaired fertility in the male animals, although histologically the sperm appeared to be normal and had normal motility (Krege et al., 1995; Esther et al., 1997). This was dependent on the loss of a testis-specific ACE isozyme that is expressed in round and elongating spermatids and was associated with defects in sperm transport in the oviduct and binding to the zona pellucida of the oocyte (Hagaman et al., 1998). In mice lacking both ACE isozymes, male fertility was selectively restored by sperm-specific expression of the testicular isoenzyme (Ramara et al., 1998).

The effects of disruption of the mouse gene encoding the AT_1 receptor types were in part predictable from the results of deleting the genes encoding angiotensinogen and ACE. Mice lacking a functional AT_{1A} receptor had a significant reduction of resting blood pressure (ca. 20 mm Hg) and lacked the pressor/depressor responses to infused Ang II that occur in normal animals. However, the *Agtr1a*(^{-/-}) animals showed no marked impairment of development and survival and had no major abnormalities in the heart, vascular system, and kidney

(Ito et al., 1995; Sugaya et al., 1995a,b; Chen et al., 1997). Closer examination of the *Agtr1a*(^{-/-}) animals revealed a slight decrease in survival, marked hypertrophy of the renal juxtaglomerular cells, and a moderate degree of mesangial expansion (Oliverio et al., 1997). Also, the tubuloglomerular feedback loop that regulates sodium delivery to the distal tubule was not detectable in AT_{1A} knockout mice, indicating a specific role of AT_1 receptor in the operation of this homeostatic mechanism (Schnermann et al., 1997). The absence of the severe renal lesions observed in animals lacking angiotensinogen or ACE at first suggested that the AT_{1A} receptor is not a critical determinant of normal renal development and structure. However, the more severe effects of angiotensinogen and ACE deficiency, and the prominent inhibitory action of losartan treatment on renal structure and function in neonatal mice, indicated that Ang II action through the AT_1 receptor is essential for normal renal growth and development (Tufo-McReddie et al., 1995).

Subsequent studies revealed that Ang II infusions in *Agtr1a*(^{-/-}) mice treated with enalapril to reduce endogenous Ang II production cause dose-related elevations in blood pressure that were prevented by AT_1 receptor antagonists. These pressor responses were much smaller than those observed in wild-type mice, but nevertheless demonstrated that AT_{1B} receptors participate in blood pressure regulation in the absence of the AT_{1A} receptor (Oliverio et al., 1997). This was confirmed by the finding that Ang II-induced calcium mobilization was similar in vascular smooth muscle cells from AT_{1A} -deficient and wild-type mice, and was blocked by losartan (Zhu et al., 1998b). Thus, the AT_{1B} receptor contributes substantially to Ang II action in the cardiovascular system in the absence of the AT_{1A} receptor, and presumably is subsidiary to the major AT_{1A} subtype in normal animals. This is supported by data obtained in mice with a double knockout of the AT_{1A} and AT_{1B} receptors, which have a more severe phenotype and lower blood pressure than mice lacking only the AT_{1A} receptor (Oliverio et al., 1998a; Tsuchida et al., 1998).

These conclusions were confirmed by the finding that mice lacking both AT_{1A} and AT_{1B} receptors showed impaired growth and marked abnormalities in renal structure and function. The renal abnormalities in the double knockout animals were similar to those seen in *Agtr*^{-/-} and *Ace*^{-/-} mice and were accompanied by comparable decreases in blood pressure and the complete absence of pressor responses to Ang II. In these animals, inhibition of converting enzyme by enalapril caused a paradoxical increase in blood pressure that could result from impairment of AT_2 receptor signaling and possibly an inhibitory effect on renal sodium excretion (Oliverio et al., 1998). These observations demonstrated that although the AT_{1B} receptor has a relatively minor role in normal animals, its contributions to growth, renal development, and cardiovascular regulation can compensate for much

of the loss of the major regulatory actions of the AT_{1A} receptors in $Agtr1a^{-/-}$ animals.

Studies on the role of the AT_1 receptors in sodium homeostasis revealed that $Agtr1a^{-/-}$ mice have a further fall in blood pressure during sodium restriction and, unlike wild-type mice, develop negative sodium balance. However, these animals showed normal increases in plasma aldosterone levels during sodium depletion, consistent with the abundance of AT_{1B} receptors in the adrenal zona glomerulosa. These findings suggest that the hypotension observed in $Agtr1a^{-/-}$ mice results from sodium deficiency and blood volume depletion and are consistent with the major role of AT_{1A} receptors in renal sodium reabsorption. The mechanisms by which Ang II regulates water homeostasis through its actions in the kidney and the brain were also clarified by observations in AT_{1A} receptor-deficient mice (Oliviero et al., 1998a,b). $Agtr1a^{-/-}$ mice have a significant defect in urinary sodium concentration and develop marked serum hypotonicity during water deprivation. This does not result from impairment of vasopressin action on water permeability in the distal tubule but from the inability to maintain a maximal sodium gradient in the kidney. This change is not increased by losartan treatment and appears to be solely dependent on AT_{1A} receptor function. In contrast, the central action of Ang II on drinking responses appears to be mediated by AT_{1B} receptors, since it is largely retained in $Agtr1a^{-/-}$ mice but is almost abolished in the mice lacking AT_{1B} receptor (Davissou et al., 1998).

In contrast to the hypertension and impaired vascular responses observed in AT_1 -deficient mice, knockout of the AT_2 receptor leads to elevation of blood pressure and increased vascular sensitivity to Ang II (Hein et al., 1995a; Ichiki et al., 1995b). This has suggested that the AT_2 receptor may exert a protective action in blood pressure regulation by counteracting AT_1 receptor function. Such an action could be exerted in part by reduced expression of the AT_1 receptor, which is increased in the vascular smooth muscle of AT_2 -deficient mice (Tanaka et al., 1998). However, the sustained hypersensitivity to Ang II in such animals is also attributable to loss of the counter-regulatory action of renal bradykinin and cyclic GMP formation (an index of NO production) (Siragy et al., 1999). The relative contributions of these two factors to AT_2 -dependent vascular regulation, and the extent to which AT_2 receptor deficiency could contribute to sustained blood pressure elevation, as in human hypertension, have yet to be determined (see Section III, D).

H. Structural Basis of Ligand Binding to the AT_1 Receptor

The cloning of Ang II receptors from several species was followed by extensive studies on the structural features that are responsible for many of the specific functional properties of the AT_1 receptor. Mutational analyses of AT_1 receptors have identified many of the amino

acid sequences and residues that are involved in the processes of ligand binding, agonist activation, G protein coupling, and internalization of agonist-receptor complexes.

1. Determinants of Ang II Bioactivity. The major features of the Ang II octapeptide (Asp^1 -Arg-Val-Tyr-Ile/Val-His-Pro-Phe⁸) that determine its biological activity were identified in early studies on the *in vivo* and *in vitro* actions of structurally modified Ang II peptides (Khosla et al., 1974). All of the biological responses that were analyzed in these early reports were mediated by what is now defined as the AT_1 receptor. These findings were extended by the development of radioligand-receptor binding assays that used radiolabeled Ang II or its peptide analogs for *in vitro* analysis of the hormone-receptor interaction. More recent studies on the properties of cloned and mutant AT_1 receptors have led to the development of ligand-receptor models that incorporate the major features currently believed to be important in agonist binding to the AT_1 receptor. They have also provided further insights into the nature of the peptide binding site and the structural features that determine receptor activation, G protein coupling, and agonist-induced desensitization and internalization of the receptor.

The biological activity of Ang II is highly dependent on the aromaticity of its Phe⁸ C-terminal residue. The aromatic side groups of Tyr⁴ and His⁶, the guanidine group of Arg⁷, and the charged carboxyl terminus, are also essential for receptor activation (Khosla et al., 1974). In contrast, the N-terminal residues are important for receptor binding and the duration of action of Ang II agonists but are not specifically required for biological activity. The Ang II (2–8) heptapeptide (Ang III) formed by deletion of the Asp¹ residue is almost as potent as the native octapeptide. The Ang II (3–8) hexapeptide (Ang IV) and the Ang II (4–8) pentapeptide also retain full biological efficacy but are weak agonists due to their low-binding affinity for the AT_1 receptor. The des-Phe⁸ Ang II (1–7) heptapeptide also binds to the receptor with low affinity, but has no agonist activity (Capponi and Catt, 1979) in most Ang II target cells, and is accordingly a weak Ang II antagonist *in vitro* (Mahon et al., 1994).

NMR analyses of Ang II and its peptide analogs yielded models for the spatial arrangement of the four pharmacophore groups, Tyr⁴, His⁶, Phe⁸, and the C-terminal carboxyl group, that determine the biologically active conformation of the peptide (Nikiforovich and Marshall, 1993; Matsoukas et al., 1994; Nikiforovich et al., 1994). These studies indicated that the three aromatic rings cluster together and suggested that a bend in the Tyr-Ile-His region of the molecule is a prominent feature of its agonistic conformation. The charged amino- and carboxyl-terminal regions of the Ang II molecule are believed to form an ion pair that maintains the hairpin shape of the Ang II molecule and also stabilizes

the binding of Ang II to the receptor. Structural comparisons of Ang II and its nonpeptide antagonist analogs by overlapping the imidazole, phenyl ring, and acidic moieties confirmed the model of Ang II as a twisted hairpin shape with about 9 Å between the His⁶ imidazole and terminal carboxylate groups (Pierson and Freer, 1992). Also, significant interactions between the Asp¹ and Arg² side chains of Ang II were suggested by proton NMR studies (Zhou et al., 1991).

Subsequent studies confirmed the compact folded shape of the Ang II peptide backbone, and the relative positions of the four pharmacophore groups. This shape resulted from electrostatic interactions between the N- and C-terminal groups and was similar to that for antibody-bound Ang II as determined by crystallography (Joseph et al., 1995a,b). The conformation of Ang II in phospholipid micelles also confirmed the hairpin structure of the molecule and suggested the presence of a stable hydrogen bond between the Phe⁸ NH and His⁶ carbonyl group, with an inverse gamma turn centered on Pro⁷ (Carpenter et al., 1998).

The Phe⁸ residue of Ang II has long been recognized to be crucial for its biological activity. This residue is important for both the binding activity and the intrinsic activity of Ang II, and even minor changes in its structure have marked effects on biological activity. Its aromaticity and steric influence determine the biologically active conformation of the C terminus of Ang II, which requires an appropriate orientation of the position 8

carboxyl group relative to the aromatic group of the phenylalanine residue. Replacement of Phe⁸ by nonaromatic residues endows antagonist properties that result from distortion of the orientation of the C-terminal carboxyl group (Aumelas et al., 1985). Consistent with its central role in receptor activation, replacement of Phe⁸ by D-Phe⁸ also caused antagonist activity, and replacement by Phe(Br5)8 causes prolonged pressor responses attributed to slow dissociation of the more hydrophobic peptide from the Ang II receptor (Bosse et al., 1990).

2. Agonist Binding Site of the AT₁ Receptor. Amino acids in the AT₁ receptor that are essential for Ang II binding include the four cysteine residues that form the two external disulfide bonds and several other residues located in the exposed surface regions of the receptor (Fig. 2). In addition, polar or charged residues located within the hydrophobic transmembrane domains, including Lys¹⁰² at the top of TM helix III and Lys¹⁹⁹ near the top of TM helix V, participate in agonist binding. Some of the extracellular residues contribute to ligand interaction and stabilization of Ang II binding and others to the conformational change that causes receptor activation. The additional disulfide bridge between the amino terminal region and the third extracellular loop of the AT₁ receptor appears to stabilize the receptor and may be necessary to maintain the proximity of the extracellular amino acids that are involved in peptide binding. Cleavage of this disulfide bond probably ac-

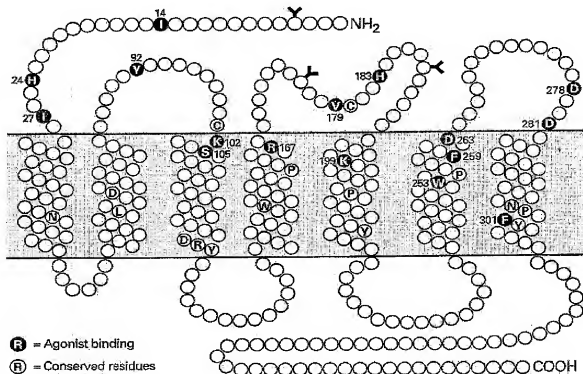


FIG. 2. Amino acids that contribute to the agonist binding site of the AT₁ receptor. Residues implicated in Ang II binding are shown as blue letters on pink background. The positions of the glycosylation sites, and of the conserved residues among GPCRs, are also shown.

counts for the impairment of AT₁ receptor binding by reducing agents (Ohyama et al., 1995).

Ang II binds primarily to the extracellular region of the AT₁ receptor (Hjorth et al., 1994) by interacting with residues in its N terminus and its first and third extracellular loops. However the transmembrane helices also participate in Ang II binding, since its C-terminal carboxyl group interacts with Lys¹⁹⁹ in the upper part of helix 5 of the receptor (Underwood et al., 1994; Noda et al., 1995a; Yamano et al., 1995). This could involve the formation of a salt-linked triad between Lys¹⁹⁹ of the receptor and the carboxyl groups of Asp¹ and Phe⁸ of the Ang II peptide (Joseph et al., 1995a,b). The Trp²⁵² residue has been proposed to stabilize the ionic bridge formed between Lys¹⁹⁹ and the carboxyl-terminal group of the Phe⁸ residue. In addition, Phe²⁵⁹ and Asp²⁶⁸ in transmembrane helix VI could provide the docking site for His⁶ of the ligand (Yamano et al., 1995). Two other residues (Lys¹⁰² and Ser¹⁰⁶) in the outer region of transmembrane helix III of the receptor have also been implicated in Ang II binding (Groblewski et al., 1995; Noda et al., 1995a). This region may participate in the formation of the intramembrane binding pocket and possibly in stabilization of the receptor's conformation.

The Asp²⁶¹ residue, located at the C-terminal end of the third extracellular loop of the AT₁ receptor, serves as a major docking point for Ang II through its charge interaction with Arg² of the ligand (Feng et al., 1995). The Asp²⁷⁸ residue could also be important in this regard, since its mutation causes an even greater loss of receptor binding affinity (Hjorth et al., 1994). The N-terminal Asp¹ residue of Ang II has been proposed to interact with His¹²³ in the second extracellular loop of the AT₁ receptor (Yamano et al., 1995). These findings support the view that Ang II attaches primarily via its charged amino-terminal end to the extracellular binding region of the receptor. The major docking points for the amino- and carboxyl-terminal ends of Ang II, Asp²⁶¹ and Lys¹⁹⁹, respectively, are located at the outer ends of helices 7 and 5, respectively. The intramembrane binding pocket lies between these proposed contact points, and is adjacent to the cleft that contains the binding sites of receptors for smaller ligands, as well as the nonpeptide binding site of the AT₁ receptor. This region contains docking sites for the apolar/aromatic mid-portion of the Ang II molecule and for the carboxyl-terminal phenyl group that elicits the conformational change(s) leading to receptor activation.

The apolar nature of the essential aromatic ring of the Phe⁸ residue of Ang II binds to the AT₁ receptor suggests that the phenyl group could interact with residues located within the membrane-spanning helices. This is consistent with NMR-based predictions of the receptor-bound conformation of Ang II that position the phenyl group in an appropriate location for such interactions (Nikiforovich et al., 1993, 1994). The phenyl group could interact with aromatic residues in the helices, some of

which have been suggested to form an aromatic floor for charge interactions between ligand and receptor (Trump-Kallmeyer et al., 1992; Findlay et al., 1993). Modeling studies on the AT₁ receptor have also suggested that conserved aromatic residues in helices IV and VI could form the base of the ligand binding site, as in other G protein-coupled receptors (Underwood et al., 1995). Mutations of two of these residues, Phe²⁵⁹ and Trp²⁶³, reduces Ang II binding to the receptor (Yamano et al., 1995). Whether the aromatic group of Phe⁸ of the ligand interacts with the binding pocket of the receptor via these amino acids, or with aromatic residues in other segments of the membrane domains, has yet to be determined. The Phe⁸ residue could also form a polar/aromatic interaction with the hydroxyl group of Ser¹⁰⁵ in the third transmembrane domain of the receptor (Joseph et al., 1995a,b).

The Tyr⁴ residue of Ang II is an important determinant of its binding and biological activities (Bumpus et al., 1977; Capponi and Catt, 1979; Nikiforovich et al., 1993, 1994). In fact, reversing the Tyr⁴ and Phe⁸ residues of the Ang II molecule (Marshall et al., 1970) formed the first Ang II antagonist. The Tyr⁴ residue has been proposed to interact with Arg¹⁰⁷ at the top of the fourth transmembrane helix (Yamano et al., 1995), and could also disrupt the hydrogen bonding between Asn¹¹¹ and Tyr²⁸² in transmembrane domains 3 and 7 of the unoccupied receptor by competing with Tyr²⁸². This would permit the latter to interact with Asp¹⁴ in the second transmembrane domain during receptor activation. In this mechanism, the loss of a proton from the Tyr⁴ phenolic hydroxyl group to the carboxyl group of Glu⁹¹ could be part of a relay system initiated by the interaction of His⁶ with Thr⁵⁶ and Glu⁹¹ of the receptor (Joseph et al., 1995a,b).

3. Antagonist Binding of the AT₁ Receptor. Since Ang II is a major regulator of blood pressure, aldosterone secretion, and fluid homeostasis, and is also an important etiological factor in hypertension and other cardiovascular disorders, blockade of Ang II formation or action by ACE inhibitors or receptor antagonists is of major therapeutic importance. Early attempt to develop therapeutic agents able to block the Ang II receptor impeded by the peptidic nature of antagonists such as saralasin, which lacked oral activity and showed agonistic properties (Pals et al., 1979). More recently, based on imidazole derivatives first described by Furukawa et al. (1982), it became possible to develop specific nonpeptide Ang II receptor antagonists that specifically and selectively block the angiotensin AT₁ receptor (Timmermans et al., 1993; Goodfriend et al., 1996). The first of this series to reach the clinic, losartan, was followed by a large number of orally active AT₁ antagonists (Table 2). These can be classified in two groups depending on the presence of a biphenyltetrazole moiety, as in the prototype drug, losartan, in their structure. Receptor binding of nonpeptide Ang II antagonists is saturable

As expected, significant increases in renin activity, Ang I and Ang II are observed after blockade of the AT₁ receptor. It is conceivable that the increased circulating Ang II level could stimulate the AT₂ receptor, which appears to counterbalance the effect of the AT₁ receptor (see below). Blockade of the AT₁ receptor not only inhibits smooth muscle contraction but also reduces the production of pressor agents including aldosterone, vasopressin, catecholamine, and endothelin. AT₁ receptor

Identification of the losartan binding region of the mammalian AT₁ receptor was facilitated by the findings that the amphibian Ang II receptor, which resembles the mammalian AT₁ receptor in its signal transduction mechanisms, does not recognize nonpeptide antagonists such as losartan. This enabled the amino acid residues involved in the binding of losartan to the mammalian AT₁ receptor to be determined by analysis of the ligand binding properties of mutant rat AT₁ receptors in which nonconserved amino acids were replaced by the corresponding amphibian residues (Ji et al., 1994). Most of these mutant receptors showed only minor changes in binding affinity for Ang II and its peptide antagonist [Sar¹, Ile⁸]Ang II, indicating that the overall conformation of the receptor was unaltered by such replacements. However, several residues located in the transmembrane domains (TMDs) of the receptor were found to be

These findings demonstrated that the nonpeptide AT₁ antagonist binds to a site defined by amino acids located within the membrane-spanning regions of the receptor. Also, the nonpeptide binding site was largely distinct from the receptor domain that is involved in binding of Ang II and other peptide ligands. This conclusion is consistent with the presence of a primordial binding site for small ligands between the transmembrane helices of all GPCRs that can be used for the development of nonpeptide analogs for a wide variety of peptide hormones. Other amino acid residues in the rat AT₁ receptor that influence losartan binding include Ala¹⁹⁸ in TMD II; Ser¹⁰⁴, Ala¹¹⁴, and Ser¹¹⁶ in TMD III; Lys¹⁹⁹ in TMD V; Phe²⁴⁸ in TMD VI; and Asn²⁹⁵ in TMD VII (Schambye et al., 1994; Ji et al., 1994; Noda et al., 1995). These and other observations have implicated TMD III in losartan binding to the mammalian AT₁ receptor. The locations of the multiple amino acids that contribute to losartan binding in the receptor are shown in Fig. 3.

A mutant amphibian receptor formed by exchanging these residues for the corresponding amino acids in the *Xenopus* AT receptor bound losartan with the same high affinity as the rat AT₁ receptor (IC₅₀ values: rat AT 2.2 ± 0.2 nM; xAT > 50 nM; mutant xAT 2.0 ± 0.1 nM) (Ji et al., 1993, 1995). This gain-of-function mutation, in

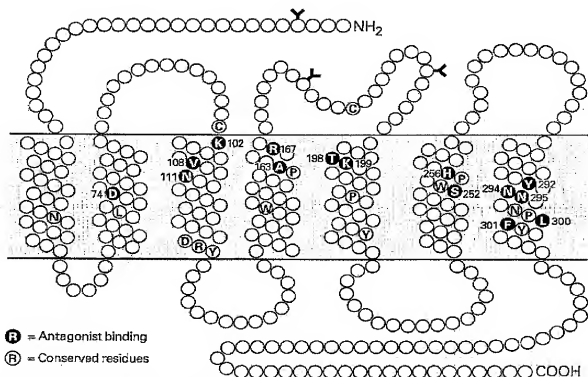


FIG. 3. Amino acids that contribute to the nonpeptide antagonist binding site of the AT₁ receptor. Residues implicated in losartan binding are indicated by green letters on orange background. The positions of the glycosylation sites, and of the conserved residues among GPCRs, are also shown.

which the residues known to participate in the formation of the ligand binding site in a mammalian hormone receptor were transferred to an unresponsive amphibian receptor, confirms the validity of the proposed losartan binding site. The identification of residues involved in receptor binding of nonpeptide Ang II analogs should aid in modeling studies on the structural basis of ligand recognition and activation of the Ang II receptor.

Although there is little overlap between the intramembrane residues that influence Ang II and losartan binding, both ligands appear to interact with the region located between helices III, V, VI, and VII of the AT₁ receptor. This area also contains the binding sites of GPCRs for small ligands such as catecholamines and acetyl choline, as well as other nonpeptide antagonist analogs. In addition to sharing the binding pocket formed by the transmembrane helices of the receptor, Ang II and its nonpeptide analogs probably interact with common contact points at other locations in the receptor. In addition to the Lys¹⁰², Ser¹⁰⁵, Arg¹⁹⁷, and Lys¹⁹⁹ residues, this could apply to Asn²⁰⁰, replacement of which by serine impairs the binding of Ang II as well as its nonpeptide agonist and peptide antagonist analogs (Hunyady et al., 1993). It is noteworthy that peptide agonist binding is also influenced by Phe³⁰¹, an aromatic residue located near the end of helix VII (Hunyady et al., 1996a, 1999). Mutation of this residue to alanine impairs Ang II and losartan binding, as well as that of the peptide antagonist, [Sar¹, Ile⁸]Ang II. These findings indicate that multiple residues contribute to the integrity of the binding pocket and are required for optimal agonist and antagonist binding.

Although the extracellular loops of the receptor contribute significantly to the binding of Ang II and its peptide antagonists, the intramembrane binding pocket has a major functional role in the AT₁ and probably most other peptide hormone receptors. In receptors for large and/or bulky ligands, the intramembrane binding pocket per se may not permit efficient interaction without additional stabilization of the ligand through binding to residues in the extracellular surface of the receptor. Nevertheless, the binding pocket is essential for the initiation of receptor activation, and its interaction of agonists triggers the change in receptor conformation that leads to G protein coupling and second messenger generation.

The importance of the transmembrane domains in receptor activation also applies to glycoprotein hormone receptors, which bind extremely large ligands. These data suggest that a common mechanism for activation of GPCRs is based on critical interactions within the intramembrane binding pocket that evoke specific changes in receptor conformation. The extracellular domains of the receptor provide additional interactions with the ligand that are necessary for high-affinity binding. This is a prominent feature of receptors for larger agonists such as glycoprotein hormones, but also occurs

in those for small peptide hormones such as angiotensin II, bradykinin, and vasopressin.

I. AT₁ Receptor Signaling Mechanisms

1. *AT₁ Receptor Activation and Signal Transduction.* The AT₁ receptor, like other GPCRs, has been proposed to undergo spontaneous isomerization between its inactive (R) and active (R*) states. The inactive R state is favored in the absence of agonist ligands and is in equilibrium with a small proportion of the active R* state in its unliganded form. In the presence of Ang II, the active R* form of the receptor is either selected or induced by agonist binding. The shift in equilibrium during agonist activation favors the R* state, in which the altered conformation of the receptor permits coupling to one or more G proteins that mediate intracellular signaling via phospholipase C and other pathways. Most receptors appear to be kept in their inactive state by structural restraints that are removed by agonist binding, leading to the formation of the activated R* state. Recent evidence suggests that the agonist-activated AT₁ receptor, and possibly other GPCRs, undergoes transitions into multiple conformational states that are associated with the individual stages of receptor activation and regulation (Thomas et al., 2000).

The proportion of receptors in the activated state varies among individual GPCRs and is manifested by varying degrees of basal signaling activity when receptors are overexpressed in transfected cells. Significant degrees of agonist-independent or constitutive activity have been observed in wild-type thyroid-stimulating hormone and muscarinic receptors (Burstein et al., 1997; Wang and Gershengorn, 1999) but are not evident in the AT₁ and most other GPCRs. Agonist-induced activation of the AT₁ receptor appears to be triggered by an interaction between the Tyr⁴ residue of Ang II and the Asn¹¹¹ residue located in the third transmembrane domain of the receptor (Noda et al., 1996). This, and the interaction of Phe⁸ of the angiotensin molecule with His²⁶⁶ in the sixth transmembrane domain of the receptor, appear to drive the AT₁ receptor into its fully active R* state (Noda et al., 1995a).

The importance of Asn¹¹¹ as a switch residue in agonist-induced AT₁ receptor activation is further indicated by the ability of its mutation to Gly¹¹¹ or other small residues to induce constitutive activation of the receptor (Peng et al., 1998). This suggests that the ability of Asn¹¹¹ to act as a conformational switch is related to its side chain size, rather than polarity or hydrogen binding. On the other hand, it is the aromaticity rather than the size of the Tyr⁴ side chain that is important in receptor activation, which probably depends on aminoaromatic binding between Tyr⁴ and Asn¹¹¹ of the receptor (Miura et al., 1999a). Aromaticity is likewise important in the interaction between Phe⁸ and His²⁶⁶, which is critical for full receptor activation by agonist ligands.

Like other constitutively activated GPCRs, the Asn¹¹¹Gly and Asn¹¹¹Ala mutant receptors show increased affinity and efficacy for agonist ligands, and exhibit marked increases in the sensitivity of their biological responses to agonist stimulation. They also discriminate between inverse agonists, which stabilize the inactive conformation of the receptor, and neutral antagonists. For these reasons, constitutively active AT₁ receptors are highly sensitive to Ang II derivatives that are weak or partial agonists at the wild-type receptor (Miura et al., 1999b).

A proposed model of GPCR activation predicts that rigid body movement of the third, sixth, and seventh transmembrane domains induces conformational changes in the cytoplasmic loops that permit G protein interaction with the agonist-activated receptor (Gether and Kobilka, 1998). However, the manner in which the activating regions in the cytoplasmic domains of individual GPCRs interact with specific subsets of G proteins is not yet known. Although many amino acids in the transmembrane and cytoplasmic regions of a wide range of receptors have been implicated in G protein coupling, there are very few cases in which individual residues can be correlated with binding to specific types of G proteins (Hunyady et al., 1996b). The limited degree of selectivity of most such residues presumably reflects the precise conformational requirements for specific interaction of receptors with their cognate G proteins.

Although the AT₁ receptor has been reported to interact with several G proteins, its major physiological functions are expressed through G_s-mediated activation of phospholipase C followed by phosphoinositide hydrolysis and Ca²⁺ signaling. Although all GPCRs possess the basic seven transmembrane structure, only a few amino acids are highly conserved among the superfamily of G protein-coupled receptors (Fig. 1). One such conserved motif is the characteristic NPX₂₋₃Y sequence that is located in the seventh transmembrane domain of most receptors, and in the rat AT_{1A} receptor is Asn²⁹⁸, Pro²⁹⁹, Leu³⁰⁰, Phe³⁰¹, Tyr³⁰². A recent model of aminergic GPCRs suggests that the NPX₂₋₃Y sequence is ideally placed to receive a signal from agonist-induced conformational changes in the ligand binding region (Donnelly et al., 1994). In the three-dimensional structure of the receptor, this sequence is in close proximity to the asparagine-aspartic acid pair located in transmembrane segments 1 and 2 and may form functionally important hydrogen bonding interactions with this region. Such models assume that the highly conserved proline residues, which disrupt the α -helical structure of the transmembrane domains, could serve as hinges during the conformational changes that occur in agonist-activated receptors. One such proline residue is located within the seventh transmembrane domain in the conserved NPX₂₋₃Y sequence and has been found to be important for signal transduction by the m₃ muscarinic receptor (Wess et al., 1993). However, this residue does not ap-

pear to be essential for signaling by Ang II and gonadotropin-releasing hormone (GnRH) receptors.

Another interesting feature of the NPX₂₋₃Y sequence is its similarity to the NPFXY internalization sequence that is present in the cytoplasmic segment of receptors for low-density lipoprotein and several growth factors. The tyrosine residue in this sequence has been reported to be essential for sequestration of the β -adrenergic receptor (Barak et al., 1994). However, this does not apply to the gastrin-releasing peptide receptor (Slice et al., 1994) and the AT₁ receptor, both of which contain an additional aromatic amino acid (Phe³²¹ and Phe³⁰¹, respectively) in their NPX₂₋₃Y sequences. The presence of such residues might be important since phenylalanine can substitute for the tyrosine residue in the NPFXY internalization sequence of nutrient receptors.

An analysis of the functional role of the Asn²⁹⁸, Pro²⁹⁹, Leu³⁰⁰, Phe³⁰¹, Tyr³⁰² sequence (Hunyady et al., 1995b) revealed that the ability of the receptor to interact with G proteins and to stimulate inositol phosphate responses was markedly impaired by alanine replacement of Asn²⁹⁸ and was reduced by replacement of Pro²⁹⁹ or Tyr³⁰². The Phe³⁰¹Ala mutant receptor exhibited normal G protein coupling and inositol phosphate responses, and the binding of the peptide antagonist, [Sar¹, Ile⁵]Ang II, was only slightly affected. However, its affinity for Ang II and the nonpeptide antagonist losartan was reduced by an order of magnitude, suggesting that Ang II and losartan share an intramembrane binding site, possibly through their aromatic moieties, in this region. None of the agonist-occupied mutant receptors, including Tyr³⁰²Ala and triple alanine replacement of Phe³⁰¹, Tyr³⁰², and Phe³⁰⁴ showed substantial changes in their internalization kinetics. These findings demonstrate that the NPLFY sequence of the AT₁ receptor is not an important determinant of agonist-induced internalization. However, the Phe³⁰¹ residue contributes significantly to agonist binding, and Asn²⁹⁸ is required for normal receptor activation and signal transduction.

Another highly conserved amino acid in the superfamily of G protein-coupled receptors is a tyrosine residue located in the fifth transmembrane helix, adjacent to the amino-terminal end of the third cytoplasmic loop. The location of this amino acid, which is Tyr²¹⁶ in the rat AT₁ receptor, suggests that it could be involved in receptor activation. The amino-terminal region of the third cytoplasmic loop adjacent to this residue has been shown to be important in the signal generation and internalization of several G protein-coupled receptors. Furthermore, modeling of G protein-coupled receptors based on the crystal structure of bovine rhodopsin has suggested that this residue is in molecular proximity to regions that are important in receptor activation, including the conserved acidic-arginine-aromatic (DRA) triplet of the second intracellular loop (Baldwin et al., 1997). Although AT₁ receptor internalization and signal transduction have different structural requirements, a

6-amino acid deletion of the amino-terminal end of the third cytoplasmic loop, which included Tyr²¹⁵, was found to prevent both receptor internalization and signaling responses. The role of the highly conserved Tyr²¹⁵ residue in the activation of GPCRs was analyzed in a mutant AT₁ receptor created by replacing this amino acid with a phenylalanine residue (Hunyady et al., 1995b).

The resulting Tyr²¹⁵Phe mutant receptor was normally expressed in transfected COS-7 cells, and its binding affinity for the peptide antagonist [Sar¹, Ile⁸]Ang II was similar to that of the wild-type receptor. However, its affinity for Ang II was significantly reduced and its ability to mediate inositol phosphate responses to Ang II stimulation was abolished. These changes were associated with loss of coupling of the mutant receptor to G proteins, as indicated by the lack of effect of GTP γ S on agonist binding to the receptor. The agonist-induced internalization of the mutant receptor was also impaired. The concomitant decreases in receptor internalization and G protein-mediated signaling of the Tyr²¹⁵Phe mutant receptor indicate that this residue has a critical role in AT₁ receptor activation. It is possible that the Tyr²¹⁵ residue takes part in propagation of the agonist-induced conformational change to the intracellular loops that participate in G protein coupling. In view of its wide conservation among members of the seven transmembrane domain receptor superfamily, this residue is likely to be of general importance in signal transduction from G protein-coupled receptors. The AT₁ receptor, like many other Ca²⁺-mobilizing GPCRs, signals primarily through coupling to a G_{q/11} protein that activates phospholipase C- β (PLC- β), leading to polyphosphoinositide hydrolysis, stimulation of InsP₃/calcium signaling, and activation of protein kinase C. However, in cultured rat VSMC it is predominantly PLC- γ rather than PLC- β that is activated during Ang II action. This response, which is dependent on tyrosine phosphorylation of the PLC- γ isozyme, is distinct from the usual process of activation of PLC- β_1 and β_2 by GPCRs through α and $\beta\gamma$ subunits derived from the heterotrimeric G proteins, G_{q/11} and G_{12/13}, respectively. AT₁ receptors have also been reported to couple to the G_{12/13} family of pertussis-insensitive G proteins, which appear to mediate Ang II-induced L-channel activation in rat portal vein myocytes (Macrez et al., 1997). In the rat, the AT₁ receptor is also coupled to pertussis-sensitive G_{i/o} protein(s) that inhibit adenylyl cyclase in several Ang II target tissues, including the adrenal glomerulosa zone, liver, kidney, and pituitary gland. Ang II has also been found to stimulate modest increases in cyclic AMP production in some of its target tissues. However, this may result from activation of Ca²⁺-sensitive adenylyl cyclases rather than coupling of the AT₁ receptor to G_i. Interestingly, although the cloned rat AT_{1A} and AT_{1B} receptors expressed in COS-7 and Y-1 cells show the expected coupling to phosphoinositide hydrolysis and other G_{q/11}-mediated responses, they do

not exhibit coupling to G_i-mediated responses such as inhibition of adenylyl cyclase (Tian et al., 1996).

2. *AT₁ Receptor and Tyrosine Phosphorylation.* In addition to its rapid actions on phosphoinositide/calcium signaling, Ang II elicits many of the intracellular signaling responses that are typically associated with activation of growth factors. These include tyrosine phosphorylation, most immediately of PLC- γ and subsequently of several other downstream proteins and effector enzymes. These include pp60^{c-src}, pp120, pp125^{FAK}, JAK2, STATs, paxillin, TYK2, and MAPK (Bernstein and Marrero, 1996). Because GPCRs do not have intrinsic tyrosine kinase activity, the ability of the AT₁ receptor to induce tyrosine phosphorylation and activation of PLC- γ in rat VSMC, with consequent increases in InsP₃ production and Ca²⁺ signaling, must depend on other tyrosine kinases. Such ligand-induced activation of PLC- γ by tyrosine phosphorylation may occur during the actions of growth factors on their target cells, and accounts for the associated elevations in [Ca²⁺]_i. This aspect of AT₁ receptor function has been controversial, because the mechanisms by which the receptor couples to PLC in VSMC are poorly defined. In most cell types, calcium-mobilizing GPCRs are coupled through their cognate G proteins to activation of the β isozymes of PLC. The β_1 and β_2 enzymes are activated by G α and G $\beta\gamma$ subunits, respectively, the former derived from G_{q/11} and the latter largely from G_{12/13} proteins. In contrast, AT₁ receptor-mediated activation of PLC- γ is dependent on tyrosine phosphorylation and consequently on signaling from receptor or nonreceptor tyrosine kinases.

The mechanism by which Ang II stimulates tyrosine phosphorylation and activation of PLC- γ involves binding of the enzyme to the C-terminal cytoplasmic domain of the AT₁ receptor. This interaction occurs between the C-terminal cytoplasmic domain of PLC- γ 1 and a phosphorylated YIPP motif located within the intracellular tail of the receptor (Venema et al., 1998a). The same YIPP motif has been implicated in the binding of JAK2 to the AT₁ receptor, probably as a complex with the phosphotyrosine phosphatase, SHP-2 (Ali et al., 1997a). The way by which AT₁ receptor activation regulates ligand-dependent binding of two distinct intracellular signaling proteins to the same site in its cytoplasmic tail has yet to be determined. The relative roles of PLC- β and PLC- γ in Ang II-induced phosphoinositide hydrolysis in VSMC have been difficult to determine, and studies on the relative abundance of the PLC isoforms in VSMC have given variable results. Relatively little PLC- β_1 was detected in rat and rabbit VSMC in some reports (Homma et al., 1993; Marrero et al., 1994), but others have found that all three isozymes (α , β , and γ) are abundant in rat VSMC (Ushio-Fukai et al., 1998). In the latter study, AT₁ receptors were proposed to couple initially to PLC- β by heterotrimeric G proteins, and subsequently to PLC- γ . The G proteins responsible for the early activation of PLC- β , and the initial maximum

InsP₃ response at 15 to 20 s, included both G_{q/11} and G₁₂. The subsequent activation of PLC- γ and its contribution to the delayed InsP₃ response are attributed to its tyrosine phosphorylation by a downstream kinase. This study also suggested that β subunits derived from G₁₂ are involved in the activation of PLC in VSMC, and mediate Ang II-induced activation of PLD in these cells.

3. *AT₁ Receptor-Activated Growth Responses.* In addition to its contractile and secretory actions in smooth muscle and adrenal cells, Ang II stimulates growth and/or proliferative responses in these and others of its target cells. This effect was first documented in rat adrenal glomerulosa cells, which respond to sodium deficiency with hypertrophy and hyperplasia, and undergo regression in animals on high sodium intake (Gross, 1968). These changes are dependent on the circulating Ang II level, which is a major determinant of trophic changes in the adrenal glomerulosa zone (Aguilera et al., 1978). Ang II also has trophic actions on renal mesangial cells and vascular smooth muscle cells, leading to cellular hypertrophy and sometimes to cell division (Huckle and Earp, 1994). Cardiac myocytes and fibroblasts also exhibit hypertrophic responses to Ang II. However, cardiac fibroblasts undergo both hypertrophy and proliferation during stimulation by Ang II, an action that contributes to the development of ventricular hypertrophy. Treatment with AT₁ antagonists has been found to abolish the growth-promoting actions of Ang II in vitro and in vivo. As discussed below, many of the growth-related actions mediated by the AT₁ receptor are inhibited by concomitant binding of Ang II to the AT₂ receptor.

The growth factor-like effects of Ang II include increases in tyrosine phosphorylation of numerous intracellular proteins, activation of MAPK and related pathways, and increased expression of several early response genes including *c-fos*, *c-jun*, and *c-myc* (Clark et al., 1992; Berk and Corson, 1997). These changes are associated with cell growth, increased thymidine incorporation, and cell proliferation (Tian et al., 1995). Such actions of Ang II are consistent with its well defined growth effects in adrenal glomerulosa and VSMC, and its role in the vascular and cardiac lesions associated with endothelial damage, atherosclerosis, hypertension, and cardiac failure. One of the earliest Ang II-induced events in VSMC is the rapid tyrosine phosphorylation and activation of PLC- γ , and the ensuing phosphoinositide/Ca²⁺ signaling responses that initiate many of the actions of Ang II in these cells (Marrero et al., 1994). This is accompanied by the rapid activation of Ras, and its dependent phosphorylation cascades that extend through the cytoplasm and into the nucleus, with consequent effects on cell growth and differentiation. These include the MAPK and related pathways, in which sequential phosphorylations that are initiated at the plasma membrane lead to subsequent actions in the nucleus and other regions of the cell.

The consequences of Ang II-induced activation of Ras in cardiac myocytes resemble those elicited by agonist activation of growth factor receptors, in which the MAPK cascade is initiated by the binding of adaptor proteins, such as Grb2 and Shc, to the autophosphorylated receptors (Sadoshima et al., 1995). These adaptors interact with guanine nucleotide exchange factors that enhance the activities of small GTP binding proteins, including Ras, Rac, and Rho. The small G proteins of the Ras superfamily are activated by recruitment of guanine nucleotide exchange factors such as Sos, which promotes the exchange of GTP for Ras-bound GDP to form the active Ras-GTP complex. Ras-GTP then recruits and activates serine/threonine kinases such as Raf-1 or Mos, which in turn activate dual-specificity MAPK kinases, such as MEKs, by serine phosphorylation. MEKs in turn phosphorylate the MAPKs, p44^{MAPK} and p42^{MAPK} (also known as extracellular signal-regulated kinases, or ERK1 and ERK2) on threonine and tyrosine residues that are located within a TEY phosphorylation motif in their activation loop. The activated MAPKs are translocated into the nucleus, where they phosphorylate other kinases and transcription factors that regulate the coordinated expression of genetic programs that control a wide variety of cellular functions. These include several early response genes and others such as *c-myc*, *Elk1*, *Ets*, *RSK*, and *Mnk*. MAPKs also exert regulatory actions on other signaling pathways in the cytoplasm and at the cell membrane. The multiple actions of activated MAPKs are terminated by a group of dual-specificity phosphatases that selectively dephosphorylate the individual MAPKs and related enzymes (Neel and Tonks, 1997).

Ang II is but one of several calcium-mobilizing GPCRs that mimic the effects of growth factors and other receptor kinases on the activation of MAPKs and early response genes. The way by which this response is linked to the well defined phosphoinositide/calcium signaling system that is used by many such receptors has only recently been established, and some aspects of this process still remain to be determined. In addition to the need for Ca²⁺ signaling (Sadoshima et al., 1995) a role of PKC was suggested by reports of phorbol ester-induced activation of Raf and Ras in certain cell types. In addition, the phorbol ester insensitive PKC isoform, PKC- ζ , was found to mediate Ang II-induced activation of ERK1/2 in VSMC and to associate with Ras during Ang II stimulation (Liao et al., 1997). Such findings indicated that additional upstream factors must be of primary importance in MAPK activation by calcium-mobilizing GPCRs. In rat VSMC, Ang II also activates Ras (Schiffer et al., 1996), an early and cardinal intermediate in stimulation of the MAPK pathway by growth factors. The Ang II-induced activation of Ras is more rapid and less prominent than that elicited by epidermal growth factor (EGF) and is substantially reduced by treatment with pertussis toxin. However, the concomitant in-

creases in MAPK activity and c-fos expression are not impaired by pertussis toxin (Okuda et al., 1996), suggesting that these responses are not dependent on the G α -mediated activation of Ras that occurs in Ang II-stimulated VSMC.

Ang II not only increases Ras-GTP levels but also stimulates the formation of Ras-Raf-1 complexes and tyrosine phosphorylation of GTPase-activating proteins (GAPs) such as p120 Ras-GAP and p190 Rho-GAP. These effects are prevented by the introduction of pp60^{src} antibodies into the cultured cells, as is the tyrosine phosphorylation of PLC- γ 1 and a substantial fraction of the Ang II-induced Ins(1,4,5)P $_3$ response (Marrero et al., 1995; Schieffer et al., 1996). These and other findings have shown that Ang II partially mimics the action of growth factors by using the Ras pathway to activate MAPK. Furthermore, this appears to involve the nonreceptor tyrosine kinase, Src (pp60^{src}), which probably acts through tyrosine phosphorylation and activation of a nucleotide exchange factor. Further evidence for the importance of Src in Ang II-stimulated activation of ERK has come from studies with tyrosine kinase inhibitors, and in Src-deficient and Src-overexpressing VSMC (Ishida et al., 1998).

4. Transactivation of Growth Factor Signaling by the AT $_1$ Receptor. In addition to Src, a calcium-dependent tyrosine kinase has been implicated in the Ang II-induced stimulation of the Ras/MAPK cascade in VSMC (Eguchi et al., 1996). This observation led to the recognition that transactivation of the EGF receptor contributes to the activation of MAPK by Ang II in these cells. This process involves the calcium-dependent phosphorylation of the EGF receptor to form docking sites for Src and the adaptor protein, Shc, which then recruits Grb2 and Sos to activate Ras (Eguchi et al., 1998).

These findings account for much of the similarity between the actions of Ang II and growth factors on the activation of PLC- γ , tyrosine kinases, and MAPKs, and increased expression of nuclear proto-oncogenes. They are also consistent with the observation that Ang and platelet-derived growth factor (PDGF) signaling cascades converge in VSMC, with activation of the PDGF- β receptor (Linseman et al., 1995). Ang II also stimulates the tyrosine phosphorylation and activation of the IGF-1 receptor, as well as insulin receptor substrate 1, in VSMC (Ali et al., 1997b). Recently, activation of the EGF receptor by Ang II has been implicated in the Ang II-induced synthesis of TGF- β and fibronectin in cardiac fibroblasts. This effect was mediated by downstream signaling from the EGF receptor, which was transactivated by Ang II via a Ca $^{2+}$ -dependent tyrosine kinase. The Ang II-induced expression of fibronectin mRNA was regulated at the transcriptional level by binding of the fos/jun complex to an AP-1 site and also by stabilization of the message through actions of TGF- β (Moriguchi et al., 1999).

These observations are relevant to the proposed role of AT $_1$ receptor activation in the remodeling of cardiac interstitial tissue during ventricular hypertrophy. Ang II has been shown to stimulate the expression of collagen and fibronectin in cardiac fibroblasts, and to promote the release of newly formed collagen from the cells (Villareal et al., 1993; Crabos et al., 1994). At least one of the calcium-dependent tyrosine kinases that could mediate the transactivation of the EGF receptor by Ang II has been identified as Pyk2 (also termed CAK β and RAFTK). However, it appears that Pyk2 does not fully account for this process and that other mechanisms are involved in AT $_1$ receptor-mediated transactivation of the EGF receptor (Murasawa et al., 1993). Another example of cross-talk from the AT $_1$ receptor is its ability to influence the activity of the LOX-1 receptor for oxidized low-density lipoprotein in human coronary artery endothelial cells (Li et al., 1999). Since oxidized low-density lipoprotein has been implicated in the pathogenesis of atherosclerosis, and attenuates nitric oxide-induced dilatation, and promotes leukocyte deposition in the vascular wall, the ability of Ang II to up-regulate LOX-1 expression via AT $_1$ receptors suggests another potential role for AT $_1$ antagonists in the management of cardiovascular disease.

5. Other AT $_1$ Receptor-Mediated Signaling Pathways. In addition to activating phosphoinositide/calcium signaling, tyrosine phosphorylation, and MAPK pathways, Ang II also acts through the AT $_1$ receptor to stimulate the Jak/STAT signaling pathway. This was first observed in neonatal cardiac fibroblasts and transfected CHO cells, in which the AT $_{1A}$ receptor activates the STAT signaling pathway and stimulates sis-inducing factor-like DNA binding activity (Bhat et al., 1994). The stimulatory effect of Ang II on SIF/STAT activation was slower than that elicited by IL-6, and was preceded by an initial inhibitory phase that was associated with concomitant suppression of IL-6-induced STAT tyrosine phosphorylation and SIF responses (Bhat et al., 1995). The inhibitory action of Ang II on IL-6-induced Stat3 signaling was attributed to activation of the MAPK pathway and could be attenuated by the MAPK inhibitor, PD98059 (Bhat et al., 1996). Ang II was subsequently found to stimulate rapid serine phosphorylation of Stat3 via a MAPK1-dependent pathway (Bhat and Baker, 1997). In neonatal cardiac myocytes, the AT $_1$ receptor is coupled through Jak2 kinase to the activation of Stat1 and Stat3, and to the formation of SIF complexes (McWhinney et al., 1997).

Ang II also causes rapid and transient activation of the Jak/STAT pathway in rat aortic smooth muscle cells (Marrero et al., 1995). This is initiated by tyrosine phosphorylation and increased catalytic activity of Jak2, and is dependent on its physical association with the agonist-activated AT $_1$ receptor. This interaction occurs at a YIPF sequence that is located in the carboxy terminal tail of the receptor (Ali et al., 1997a,b), and is also

present in the PDGF receptor. This motif has also been implicated in the binding of SH2 domains in PLC- γ to the AT₁ receptor (Venema et al., 1998a). The binding of Jak2 to this motif has been attributed to the participation of SHP-2 phosphotyrosine phosphatase, which also contains an SH2 domain and functions as an adaptor protein in the association between JAK2 and the AT₁ receptor (Marrero et al., 1998).

In addition to ERK1 and ERK2, Ang II stimulates the activity of c-Jun N-terminal kinase (JNK) members of the MAPK family. In vascular smooth muscle cells, p21-activated kinase (PAK), which mediates JNK activation by IL-1 and tumor necrosis factor, was rapidly stimulated by Ang II before the activation of JNK. Ang II-induced activation of both PAK and JNK was dependent on calcium and PKC, and partially on a tyrosine kinase other than Src. These findings, and the ability of a dominant negative PAK to attenuate the JNK response to Ang II in transfected CHO and COS cells, indicated that PAK is a mediator of JNK activation by Ang II in VSMC (Schmitz and Berk, 1997). In this regard, Ang II again behaves like inflammatory cytokines such as IL-2 and tumor necrosis factor- α , by acting through PAK as an upstream mediator of JNK.

Ang II also promotes hypertrophy of VSMC through an oxidant stress-dependent mechanism. Reactive oxygen species such as hydrogen peroxide and O₂⁻ appear to be involved in the pathogenesis of hypertension and atherosclerosis, and are released from endothelial cells and other vascular and circulating cells (Rajagopalan et al., 1996). These molecules have been implicated in vascular smooth muscle proliferation and in the development of hypertension (Laursen et al., 1997; Abe and Berk, 1998). The reactive oxygen species that are generated by xanthine oxidase are potent stimuli of VSMC proliferation (Rao and Berk, 1992). Conversely, antioxidants reduce the cell response to growth factors, and inhibit the proliferation of VSMC (Boscoboinik et al., 1995). In cultured rat VSMC, Ang II causes rapid increases in intracellular H₂O₂ and phosphorylation of p42/44 MAPK and p38 MAPK. Treatment with H₂O₂ activates only p38 MAPK, and an NADH/NADPH oxidase inhibitor reduced only p38 MAPK phosphorylation in Ang II-treated cells. Also, blockade of Ang II-induced p38 MAPK phosphorylation by transfected catalase inhibited solely p38 MAPK phosphorylation, and reduced Ang II-induced cell hypertrophy. These findings indicate that both the p38 MAPK and the p42/44 MAPK pathways are required for the hypertrophic response of VSMC to Ang II (Ushio-Fukai et al., 1998).

In addition to their role in Ang II-induced activation of p38 MAPK, reactive oxygen species mediate the activation by Ang II of Akt/protein kinase B (Akt/PKB) in VSMC (Ushio-Fukai et al., 1999b). This serine-threonine kinase has been implicated in protein synthesis and also appears to be an important component of a survival pathway that protects cells from apoptosis. Akt/PKB is

activated by several growth factors via Ras and phosphatidylinositol-3 kinase, and also by heat shock and other stresses. In rat VSMC, the activation of Akt/PKB by Ang II is dependent on tyrosine phosphorylation and the activity of its upstream effector molecule, phosphatidylinositol-3 kinase (Takahashi et al., 1999). These findings suggest that Akt/PKB has an important role in the intracellular actions of Ang II and in particular in its effect on cell survival (Pollman et al., 1996).

NO inhibits several of the physiological actions of Ang II, and prevents its activation of ERK, JNK, and p38 MAPK (Wang and Murphy, 1998). In rat cardiac fibroblasts, NO inhibits the activation of PYK2 and causes a concomitant decrease in ERK phosphorylation in Ang II-stimulated cells. Since PYK2 is essential for Ang II-induced activation of ERK in VSMC (Sabri et al., 1998), these findings suggest that it could be a locus at which NO regulates calcium-dependent signaling by the AT₁ and other G α -coupled receptors (Lev et al., 1995).

J. Receptor Activation and Endocytosis

Agonist activation of many plasma-membrane receptors is followed by clustering of the complexes in clathrin-coated pits, and subsequent internalization in clathrin-coated vesicles to undergo degradation or recycling to the plasma membrane. Although nutrient receptors (e.g., for low-density lipoprotein and transferrin) frequently exhibit constitutive internalization, the hormone and growth factor receptors that evoke intracellular signals usually undergo ligand-induced endocytosis. Growth factor receptors with intrinsic tyrosine kinase activity and G protein-coupled receptors are internalized by a similar clathrin-coated vesicular endocytic process. There is increasing evidence that the intracellular signaling mechanisms that are activated by these receptors do not necessarily participate in the internalization process. Such a dissociation between Ang II-induced G protein activation and receptor internalization was observed in studies on mutant and wild-type AT_{1A} receptors expressed in COS-7 cells (Hunyady et al., 1994b). Mutations of the third cytoplasmic loop revealed that the N-terminal part of this region is important for both receptor endocytosis and intracellular signaling. Also, three point mutations of the conserved Asp⁷⁴ residue in TMD II, which has been implicated in signal transduction by the AT_{1A} receptor and other GPCRs (Bihoreau et al., 1993), significantly impaired G protein coupling and phosphoinositide hydrolysis. However, the Asp⁷⁴ (D74N, D74H, and D74Y) showed markedly different internalization kinetics. The D74Y receptor showed the greatest impairment of internalization but retained the highest degree of inositol phosphate stimulation. In contrast, the D74N mutant, which showed the most impaired G protein coupling and inositol phosphate responses, had normal internalization kinetics. The combined mutant receptor containing the D74N substitution and deletion of residues 221–226 from the third cytoplasmic loop

showed no G protein coupling or inositol phosphate response but was internalized about 60% as rapidly as the wild-type receptor. These data demonstrate that endocytosis of the AT_1 receptor is independent of agonist-activated signal transduction and indicate that receptor internalization and activation phospholipase C have different structural requirements.

The demonstration that coupling to heterotrimeric G proteins and intracellular signaling responses is not required for internalization of the AT_1 receptor indicated that the conformational changes that result from agonist-induced receptor activation exert largely independent effects on signal transduction and receptor endocytosis. However, since the structural requirements for signaling and internalization overlap, mutations that cause changes in G protein coupling are frequently accompanied by impairment of endocytosis. Thus, the N-terminal region of the third cytoplasmic loop is important for both receptor signaling and internalization. However, the ability of internalization-deficient mutant receptors with specific changes in the cytoplasmic tail or third loop to activate their respective G proteins and signaling responses indicates that G protein activation alone is not sufficient to induce receptor endocytosis. The sequences in these regions that are important for receptor internalization are frequently enriched in serine and threonine residues. For example, the rat AT_1 receptor contains 12 serine/threonine residues in the 23-amino acid segment located between residues 326 and 348 of the cytoplasmic tail (Fig. 4).

A mutational analysis of this region of receptor revealed that deletion of the carboxyl-terminal 22 amino acids of the receptor did not affect agonist-induced endocytosis (Hunyady et al., 1994a). However, internalization was markedly impaired by removal of one additional residue (Leu³⁸⁷) and was reduced by 95% after removal of the adjacent Ser³³⁵ and Thr³³⁶. In addition, triple alanine replacement of the Ser-Thr-Leu residues reduced internalization to almost the same extent as the corresponding tail deletion mutant. The Ser-Thr-Leu motif is highly conserved in mammalian AT_1 receptors but is not present in the noninternalizing AT_2 receptor. These findings demonstrate that a serine/threonine-rich region including Leu³⁸⁷ in the cytoplasmic tail of the AT_1 receptor is a major requirement for endocytosis of the

hormone-receptor complex, and support the concept that similar motifs in other G protein-coupled receptors are determinants of their agonist-induced internalization. An additional region in the cytoplasmic tail of the AT_1 receptor, involving hydrophobic and aromatic residues on a putative α -helix adjacent to the cell membrane, has also been found to be involved in agonist-stimulated endocytosis (Thomas et al., 1995). It is possible that these two regions in the cytoplasmic tail function cooperatively to trigger endocytosis, and that agonist-induced receptor activation leads to phosphorylation of the serine/threonine-rich region and causes a conformational change that exposes the more proximal hydrophobic residues for interaction with the endocytic mechanism.

K. AT_1 Receptor Function in Selected Tissues

1. *The AT_1 Receptor and the Brain.* Ang II regulates numerous physiological responses through its central actions in the brain, where it functions as a neurotransmitter or neuromodulator to influence blood pressure, drinking behavior, salt appetite, and several neuroendocrine processes. Some of these responses are induced by the actions of circulating Ang II at the circumventricular organs and other specialized regions, and others are influenced by locally formed Ang II generated within the brain itself. Although circulating hormones are effectively excluded from most parts of the brain by the blood-brain barrier, neurons in the circumventricular organs are accessible to many circulating ligands via the fenestrated endothelial cells of their dense capillary circulation. Due to the absence of the blood-brain barrier at these sites, neurons within the subfornical organ (SFO), organum vasculosum lamina terminales (OVLT), and area postrema are exposed to circulating hormones, ions, and other potential regulatory factors. These three structures, sometimes called the sensory circumventricular organs (Johnson and Gross, 1993), are rich in AT_1 receptors and have been implicated in numerous homeostatic processes. Ang II is but one of several peptides that bind to high-affinity receptors located on circumventricular neurons that project to hypothalamic nuclei and other brain regions. Other ligands with abundant circumventricular receptors include atrial natriuretic peptide, vasopressin, cholecystokinin, GLP-1, calcitonin, and relaxin (McKinley and Oldfield, 1993).

The pressor response to circulating Ang II is mediated by the area postrema and the SFO, and its stimulatory actions on water and salt ingestion, vasopressin secretion, and adrenocorticotropin (ACTH) release are mediated by the SFO. AT_1 -expressing neurons in the SFO send projections to the supraoptic and paraventricular nuclei of the hypothalamus to influence vasopressin and ACTH release (Oldfield et al., 1994), and to the median optic nucleus to stimulate water drinking (Cunningham et al., 1991).



Fig. 4. Location of agonist-induced phosphorylation sites in the AT_1 receptor. The potential serine and threonine phosphorylation sites are shown in bold letters. The most highly phosphorylated region in the C-terminal cytoplasmic tail during Ang II action is indicated by the shaded box. Potential PKC sites are indicated by brackets, and the corresponding phosphorylated serines are shown by asterisks. Recent evidence suggests that low agonist concentrations selectively induce PKC phosphorylation by other kinases is predominant at higher agonist concentrations (Quin et al., 1999).

Like several other peptide hormones, Ang II exerts effects in the central nervous system that are complementary to its primary physiological actions in peripheral tissues. This is well exemplified by the presence of brain AT_1 receptors at sites that influence cardiovascular function, fluid and electrolyte homeostasis, and pituitary hormone secretion (Allen et al., 1998, 1999). The existence of an endogenous brain renin-angiotensin system that is distinct from the systemic system comprised of the kidneys, liver, and lungs has been demonstrated by numerous studies over the last 30 years. Early evidence for a central mechanism that contributes to the development of Ang II-induced hypertension (Bickerton and Buckley, 1961) was followed by the demonstration of renin and angiotensin II in the brain (Fisher-Ferraro et al., 1971). These and other studies led to the recognition of an autonomous brain renin-angiotensin system that has significant actions in cardiovascular regulation and several other brain functions (Ganten and Speck, 1978; Ganong, 1984; Phillips, 1987).

The ability of centrally administered Ang II to increase blood pressure, fluid intake, and vasopressin release presaged the existence of specific receptors in the brain regions controlling these functions. This was confirmed by the selective effects of Ang II on brain structures that influence blood pressure, sympathetic nerve activity, drinking behavior, salt appetite, and pituitary hormone secretion (Phillips, 1987; Ganong, 1993; Wright and Harding, 1994). Effects of angiotensin on learning and memory, as well as sensory function, have also been observed. The number and scope of these central effects of angiotensin indicate that the renin-angiotensin system has a major role in the regulation of neural components that control multiple physiological functions. Most of these actions are mediated by AT_1 receptors, which are expressed in brain regions known to be involved in the control of cardiovascular function and body fluid homeostasis.

At the cellular level, direct application of Ang II increases the firing rate of neurons in the supraoptic nucleus, subfornical organ, and paraventricular nucleus (Nicol and Baker, 1971; Felix and Akert, 1974; Ambuhl et al., 1992a,b) as well as in the nucleus tractus solitarius, dorsal motor nucleus of the vagus, and thalamus (Wayner et al., 1973; Felix et al., 1988). In several of these studies, the angiotensin II (2–7) heptapeptide (Ang III) was found to be as or more effective than Ang II, raising the possibility that Ang III may be the centrally active form of Ang II (Harding et al., 1986). The application of topical autoradiography to receptor mapping in tissue sections revealed that angiotensin II receptors are present in many of the brain nuclei and other regions that are known to be involved in the central regulation of cardiovascular regulation and fluid homeostasis (Mendelsohn et al., 1984). The distributions of Ang II receptors in the brains of several mammalian species have been mapped by topical autoradiography

with radioiodinated ligands, usually ^{125}I -[Sar¹, Ile⁶]Ang II. Specific AT_1 and AT_2 receptors were identified by inhibition of radioligand binding by selective AT_1 (losartan) and AT_2 (PD123177) receptor antagonists. The distribution of AT_1 receptor messenger RNA in the rodent brain has also been analyzed by *in situ* hybridization using specific riboprobes to detect AT_{1A} and AT_{1B} receptor subtypes (Lenkei et al., 1998). Such studies have shown that all of the well defined physiological actions of Ang II in the brain are mediated by AT_1 receptors.

In all species studied, AT_1 receptors are most abundant in the hypothalamus and the circumventricular organs. In the hypothalamus, AT_1 receptors are localized to the parvocellular region of the paraventricular nucleus, which has been implicated in the control of anterior pituitary hormone secretion, ingestive behavior, and autonomic regulation of the cardiovascular system. In contrast, AT_1 receptor expression is low or absent in the magnocellular neurons of the paraventricular nucleus that control the release of vasopressin and oxytocin from the neurohypophysis. The circumventricular organs, which include the median eminence, arcuate nucleus, SFO, and OVLT, contain high densities of AT_1 receptors. At each of these sites, the local deficiency in the blood-brain barrier renders them accessible to Ang II and other regulatory ligands in the circulation. Together with the interposed median preoptic nucleus, which also contains abundant AT_1 receptors, the SFO and OVLT comprise the lamina terminalis of the forebrain. This structure has neural connections with the parvocellular neurons of the hypothalamus, as well as efferent projections to the supraoptic nucleus that subserve vasopressin secretion and other osmoregulatory responses including sodium excretion and thirst (McKinley et al., 1992).

In contrast to the relatively wide distribution of AT_{1A} receptor expression in the CNS, the AT_{1B} receptor is not detectable in most brain areas. However, AT_{1B} receptors are the predominant subtype expressed in the anterior pituitary gland, where they mediate the direct actions of Ang II on pituitary function. Both Ang II and Ang III can elicit a variety of physiological changes, including pressor and dipsogenic responses, and increased salt appetite, when injected into the cerebral ventricles (Wright and Harding, 1992). This is accompanied by increased firing of magnocellular neurons in the supraoptic nucleus (Okuya et al., 1987; Yang et al., 1992). Injection of Ang II or Ang III into the supraoptic and paraventricular nuclei also stimulates magnocellular neurons, leading to the release of vasopressin from the posterior pituitary gland (Phillips, 1987).

2. Ang II-Induced Neuronal Signaling Pathways. AT_1 receptor-mediated signaling pathways in neurons are generally similar to those observed in other Ang II target cells. In addition to phosphoinositide hydrolysis, with formation of $InsP_3$ and diacylglycerol, elevation of $[Ca^{2+}]_i$, and activation PKC, Ang II stimulates the *ras*/

raf/MAPK cascade and translocation of MAPK into the nucleus (Lu et al., 1996). It also increases the expression of *c-fos*, *c-jun*, and other early response genes. The MAPK pathway mediates Ang II-stimulated norepinephrine synthesis and reuptake mechanisms in cultured neurons (Yang et al., 1996). In addition, Ang II-induced increases in $[Ca^{2+}]_i$ activate calcium/calmodulin protein kinase II, which acts together with PKC to mediate the rapid actions of Ang II on neuronal membrane currents. These include a PKC-mediated increase in total neuronal Ca^{2+} current (I_{Ca}), as well as PKC- and calcium/calmodulin protein kinase II-mediated inhibition of delayed rectifier K^+ current (K_v) and transient K^+ current (I_A) (Zhu et al., 1997a, 1998a). In rat supraoptic neurons, AT_1 receptors mediate Ang II-induced activation of a nonselective Na^+/Ca^{2+} channel that promotes neuronal depolarization (Yang et al., 1992). Some of these effects of AT_1 receptor activation in neurons, including MAPK activation, are under the inhibitory control of AT_2 receptors via activation of serine/threonine type 2A (PP-2A) (Huang et al., 1996) and possibly tyrosine phosphatases such as MAPK phosphatase.

3. Role of Ang III in the Brain. Ang II is rapidly cleared from the circulation with a half-life of 13 s and is extensively metabolized in its target tissues to form Ang III, Ang IV, and Ang (4-8) (Harding et al., 1986; De Silva et al., 1988). Although the Ang II octapeptide is the major active species acting at AT_1 receptors in peripheral Ang II target tissues, the possibility that Ang III could be the predominant effector peptide in the brain has been recognized for several decades. This was first suggested by the observation that Ang III was twice as effective as Ang II in stimulating the firing rate of SFO neurons (Felix and Schlegel, 1978). This finding, and similar observations in paraventricular nucleus neurons, led to the suggestion that Ang III could be the centrally active form of Ang II (Harding et al., 1986). This proposal gained support from studies using nonselective aminopeptidase inhibitors such as bestatin, which increased the half-lives of Ang II and Ang III in the brain and potentiated the stimulatory action of Ang II and Ang III on paraventricular neurons (Harding and Felix, 1987).

The recent development of specific inhibitors of aminopeptidase A (APA) and aminopeptidase N (APN), which hydrolyze the N-terminal residues of Ang II and Ang III, respectively, has facilitated the investigation of the roles of Ang II and Ang III in the brain. The effects of i.c.v. administration of these compounds indicated that the central action of Ang II on vasopressin release in mice depends on its prior conversion to Ang III, confirming that Ang III acts as a major effector peptide of the renin-angiotensin system in the brain (Zini et al., 1996). Furthermore, the ability of centrally administered Ang II to stimulate the firing rate of magnocellular vasopressinergic neurons of the supraoptic nucleus

(SON) of the rat was prevented by central administration of the selective APA inhibitor, EC33. Since the SON does not contain AT_1 receptors, this effect of EC33 presumably results from its inhibition of APA in the subfornical organ, from which Ang II-sensitive neurons project to the SON (Zini et al., 1998).

Recent studies on the effects of selective APA and APN inhibitors on blood pressure in the rat have indicated that Ang III is also the main effector peptide of the brain RAS in the regulation of blood pressure (Reaux et al., 1999). Central inhibition of APA by EC33 not only blocked the pressor action of Ang II but also caused a fall in basal blood pressure comparable to that caused by i.c.v. administration of losartan, indicating a requirement for Ang III in the tonic regulation of blood pressure. Furthermore, the inhibition of APN activity by PC18 caused an increase in blood pressure that was blocked by losartan. This suggests that inhibition of Ang III degradation by PC18 causes an increase in Ang III levels that elevates blood pressure through an action on AT_1 receptors. These observations indicate the potential applications of APA inhibitors as centrally acting compounds in the management of hypertension.

These observations raise the question of whether the central actions of Ang III are mediated by an as yet unidentified AT_1 -type receptor with higher affinity for Ang III over Ang II. This seems unlikely in view of the probability that such a receptor with similarity to the AT_1 site would have been detected during isolation and cloning of the AT_1 and AT_2 subtypes. The alternative proposal, that blockade of the conversion of Ang II to Ang III by APA favors the activation of other Ang II-degrading pathways, appears to be a more likely explanation.

4. The AT_1 Receptor and the Pituitary Gland. The presence of Ang II receptors in the anterior pituitary gland was suggested by early reports of actions of the octapeptide on pituitary hormone secretion (Steele et al., 1981). This was later confirmed by binding studies with ^{125}I -Ang II, which demonstrated specific, high-affinity binding sites in the anterior pituitary glands of rat, rabbit, and dogs. No receptors were found in the posterior pituitary gland or in GH3 pituitary tumor cells (Hauger et al., 1982; Mukherjee et al., 1982). The sites were nanomolar affinity, and bound the heptapeptide des-Asp¹-Ang II with about one-tenth the affinity of the octapeptide. Ang II and the heptapeptide showed a similar potency ratio in their ability to stimulate ACTH release from pituitary cells (Capponi et al., 1982). Fractionation of rat pituitary cells revealed that Ang II receptors were associated with lactotrophs and stimulated prolactin as well as ACTH release. These findings suggested that Ang II contributes to the physiological control of prolactin secretion (Aguilera et al., 1982).

The stimulatory action of Ang II on prolactin production by pituitary lactotrophs is mediated by the AT_1 receptor, which is coupled to phosphoinositide hydroly-

sis and PKC activation, and also affects cyclic AMP production (Audinot et al., 1991). In addition to these stimulatory actions, Ang II also exerts AT_1 -mediated inhibitory actions on adenyl cyclase activity in pituitary cell membranes. In intact cells, blockade of AT_1 receptors by losartan prevented AT_1 -induced prolactin release, as well as the phospholipase C and PKC-dependent increase in cyclic AMP production (Moreau et al., 1994). During development the responsiveness of rat pituitary lactotrophs to Ang II increases with age, and by 28 days was more prominent in female than in male rats (Diaz-Torga et al., 1994). The ability of the hypothalamic GnRH to stimulate prolactin release in perfused pituitary cells, but not gonadotropin release, was abolished by losartan, consistent with a paracrine role of Ang II in GnRH-stimulated prolactin release (Becu-Vilalobos et al., 1994). Interestingly, the ability of Ang II to stimulate prolactin release from cultured pituitary cells from animals fed on an essential fatty acid-free diet was markedly decreased, although the stimulatory action of thyrotropin-releasing hormone, and the inhibitory action of dopamine were not affected (Alessio et al., 1994).

Ang II also increases the proliferation of cultured mammatrophs, and this effect is suppressed by tamoxifen and restored by estradiol-17 β . Treatment with the peptide Ang II antagonist, saralasin, inhibited not only the Ang II-stimulated proliferative response but also that of GnRH, again indicating that GnRH-stimulated release of Ang II, together with estradiol, regulates lactotroph proliferation in the rat pituitary gland (Shinkai and Ooka, 1995). The stimulatory action of Ang II on prolactin release was associated with a rapid increase in intracellular calcium concentration that was abolished by losartan. However, in the same report no increase in [3H]thymidine incorporation was observed (Diaz-Torga et al., 1998). An analysis of the distribution of the AT_1 receptor subtypes in the rat anterior pituitary gland, which expresses predominantly (80%) AT_{1B} receptors, revealed that the majority of the AT_{1B} receptor-expressing cells are lactotrophs, and the remainder are corticotrophs. In adult male rats, AT_{1B} receptors are located in about 50% of the pituitary lactotrophs and about 25% of the corticotrophs. These findings suggest that Ang II synthesized in gonadotrophs can directly stimulate prolactin and ACTH release from lactotrophs or corticotrophs through activation of AT_{1B} receptors (Lenkei et al., 1999). This AT_{1B} -mediated paracrine mechanism in the pituitary gland is subsidiary to its endocrine regulation by AT_{1A} -mediated changes in the hypothalamic production of releasing hormones and neurotransmitters including dopamine and catecholamines (Denef, 1986; Ganong, 1993).

5. The AT_1 Receptor and the Heart. The role of the renin-angiotensin system in cardiovascular control and body water homeostasis is well documented (reviewed in Saavedra, 1992; Wright and Harding, 1994, 1997; Höhle

et al., 1995; Mosimann et al., 1996; Unger et al., 1996). Ang II acts primarily at the AT_1 receptor type in a variety of tissues including vascular smooth muscle, kidney, and adrenal gland to influence vasoconstriction and sodium reabsorption. Furthermore, it is known that Ang II acts locally within cardiac tissues to influence protein synthesis and cellular growth (Baker and Aceto, 1990; Lindpainter and Ganten, 1991; Baker et al., 1992; Dostal and Baker, 1992; Dostal et al., 1992, 1996). There is accumulating evidence that the AT_1 receptor may be involved in the development of cardiac hypertrophy via the regulation of extracellular matrix accumulation (Weber and Brilla, 1991; Weber et al., 1993, 1995a,b; Brilla et al., 1993, 1995b). Specifically, cardiac fibroblast stimulation appears to facilitate the accumulation of collagen in the extracellular matrix of the heart (Brilla et al., 1995a). Furthermore, Ang II appears to block MMP-1 activity, an enzyme directly involved in the degradation of fibrillar collagen. Thus, interference with MMP-1 results in a proliferation of extracellular matrix. Ang II-induced hypertrophy in cardiac myocytes and vascular smooth muscle cells follows the pattern of an initial increase in protein synthesis and cell size without an elevation in numbers of cells (Geisterfer et al., 1988; Berk et al., 1989). After prolonged exposure to Ang II, mitogenic changes were also observed (Weber et al., 1994). The ability of ACE inhibitors and AT_1 receptor antagonists to prevent the onset of cardiac hypertrophy in animal models (Zhu et al., 1997b), and to promote its regression in hypertensive patients (Thürmann et al., 1998), has also demonstrated the role of AT_1 receptors in ventricular hypertrophy. These agents also prevent the onset of ventricular dilatation in rats after myocardial infarction by suppressing the expression of gene expression (ANF, β -myosin heavy chain, collagen) associated with cardiac remodeling (Yoshiyama et al., 1999). To determine whether this effect results from inhibition of the direct action of angiotensin on the heart, or reduction of pressure overload, Paradis et al. (2000) created transgenic mice over-expressing the AT_1 receptor in cardiac myocytes. These animals spontaneously developed cardiac hypertrophy and remodeling, with increased expression of cardiac ANF and interstitial collagen deposition. They showed no change in heart rate or blood pressure, but died of heart failure at an early age. These findings indicate that the AT_1 receptor mediates the direct actions of Ang II in the development of ventricular hypertrophy and heart failure. However, it should be noted that some of the beneficial effects of ACE inhibitors and AT_1 antagonists on cardiac function could result from activation of B_2 kinin and AT_2 receptor-dependent pathways that modulate myocyte contractility and growth (Wollert and Drexler, 1999). The role of the bradykinin B_2 receptor remains, however, disputed (Mad-eddu et al., 2000).

III. The Type 2 (AT₂) Angiotensin Receptor

Whereas differential stability to dithiothreitol (Chang et al., 1982; Gunther, 1984; Chiu 1989b; Speth et al., 1991) suggested the presence of more than a single form of angiotensin II binding or receptor site (Chiu et al., 1989a; Whitebread et al., 1989; Chang and Lotti, 1990), cloning and expression of the receptor types AT₁ (Murphy et al., 1991; Sasaki et al., 1991) and AT₂ (Kambayashi et al., 1993b; Mukoyama et al., 1993) provided clear evidence for the presence of the second isoform, AT₂. The identification and characterization of the biochemical and physiological functions of the AT₂ receptor is still a matter of intense research.

A. Cloning, Purification, and Properties of the AT₂ Receptor

The AT₂ receptor is clearly distinct from the AT₁ receptor in tissue-specific expression, signaling mechanisms, and diversity in molecular weight. In the early phase of AT₂ receptor research it was suspected that the AT₂ receptor may not even be a G protein-coupled seven transmembrane domain receptor (Bottari et al., 1991). Clearly, its structure had to be determined by cloning and purification. Whether it was a single entity or had related isoforms was not apparent in the beginning. Undoubtedly, more fundamental studies were needed,

which should be based on the cloning of the AT₂ cDNA and purification of the AT₂ receptor.

There are cell lines expressing the AT₂ but not the AT₁ receptor, such as PC12W, R3T3, and some lines of N1E-115. Fetal rats express a large amount of the AT₂ receptor protein and its mRNA. However, the cloning of the AT₂ receptor turned out to be difficult. Attempts to screen cDNA libraries with AT₁ cDNA as a probe did not work, indicating a lack of homology between the AT₁ and AT₂ receptor gene. Thus, the expression cloning method had to be used. For some unknown reason, cDNA detected by autoradiographic identification of transfected COS-7 cells presumably expressing AT₂ mRNA could not be recovered from these cells. After several technical refinements, a 4.5-kb rat AT₂ cDNA was cloned from rat PC12W cells by Kambayashi et al. (1993b), and from rat fetuses by Nakajima et al. (1993). It contained seven hydrophobic sequences compatible with the structural theme of the seven transmembrane domain receptors super family (or GPCR) (see Fig. 5) but showed only a 32% amino acid sequence identity with the rat AT₁ receptor.

Since targeted gene deletion can be performed only in the mouse, Ichiki et al. (1994) and Nakajima et al. (1993) cloned mouse AT₂ cDNA from a fetal mouse cDNA library by a plaque hybridization method using rat AT₂

RAT ANGIOTENSIN RECEPTOR AT₁ AND AT₂

AT ₁		MAIN	SSAEDGKRI	QDDCPKGRH	SYIEVMIPTL	34
AT ₂	MDNFSFAAT	SRNITSSLPF	DNLNATGTNE	SAFNCSHKPA	DRHLEAIPVL	80
AT ₁	YSIIFVVGIF	QNSLVVIVIX	FYMKLKFTAS	VFLNLALAD	LCFLTLPLPW	84
AT ₂	YMIIFVIGTA	VNIIVVSLFC	CQKGPVKVSS	TYIENLAAD	LLLTALPLW	100
TM-1						
AT ₁	AVYTAMEYRW	PFGNHLCKIA	SASVTFNLYA	SVFLLTCLSI	DRYLAIVHEM	134
AT ₂	ATYYSRYRDW	LFGFVMCKVF	GSELTIANMFA	SIFFTITCMV	DRYQSVIYFF	150
TM-2						
AT ₁	KSRLRRITLV	AKVTCIIWL	MAGLASLPV	IHRNVYFIEN	TNITVCAFHY	184
AT ₂	LSQRNP-WQ	ASVVPFLVWC	MACLSSELEF	YFQDVRIEY	LGVNACIMAF	199
TM-3						
AT ₁	ESRNSLTPIG	LGLT-KNIIIG	FLFPPLIIIT	SYTLIWKALK	KAYEIQKNKP	233
AT ₂	PPEKYAQWSA	GIALMKNIIG	FIIPLIFITAT	CYFGIRKHL	KINSYQGNRI	249
TM-4						
AT ₁	RNDIDIERIIM	AIVLFFFSW	VPHQIFTEFD	VLIQLGVHID	CKISDIDVTA	283
AT ₂	TRDQVLRMAA	AVVLAFTICW	LFPHVLTFLD	ALTWMGLINS	CEVIAVIDLA	299
TM-5						
AT ₁	MPITICIAEYF	NNCINPLFYC	FLGKKEKKYF	LQLLKXIPPK	AKSHSSLSSTK	323
AT ₂	LPFAILIGET	NSCVNFFLYC	FVGNRFQQL	RSVFRFPIITW	LQKKRETMSC	349
TM-6						
AT ₁	MSTLSYRPSD	NMSSSAKKPA	SCFEVE			359
AT ₂	RKSSSIREMD	TFVS				363

FIG. 5. Amino acid sequences of rat AT_{1A} and AT₂ deduced from respective cDNA. Sequences in shaded boxes are identical. Of the overall 92% sequence identity, the transmembrane regions show a somewhat higher degree of homology.

cDNA as a probe. A mouse AT_2 genomic 4.4-kb DNA fragment was cloned by Ichiki et al. (1994) and Koike et al. (1994). Since these studies show that the entire coding sequence is contained in one exon, the human AT_2 coding sequence (Tsuzuki et al., 1994) or fragments containing more extensive sequences could be cloned (Martin et al., 1994; Koike et al., 1994). The genomic DNA of all three species consist of three exons with an uninterrupted coding region being confined to the third exon. The genes encoding the AT_2 receptor are localized in human chromosome Xq22-q2, rat chromosome Xq3, and mouse chromosome X (Koike et al., 1994; Hein et al., 1995b; Tissir et al., 1995). The localization in the long arm of the X chromosome throughout species may have important implications in targeted gene deletion as well as in the genetics of the AT_2 receptor gene.

The open reading frame of AT_2 cDNA encodes 363 amino acid residues in all three species with 99% sequence identity between rat and mouse and 72% identity between rat and human. The divergence between rodent and human AT_2 occurs mainly in the N-terminal region. AT_1 and AT_2 receptors have only 33 to 24% amino acid sequence identity (Kamhayashi et al., 1993b; Nakajima et al., 1993). The homology was mainly localized in the transmembrane hydrophobic domains, which are believed to form seven transmembrane helical columns. Residues located in these helical column domains and considered to be essential for Ang II binding to the AT_1 receptor are also preserved in the AT_2 receptor. These include Lys¹¹⁸ (Lys¹⁰² of AT_1) at the top of TM3, Arg¹⁸³ (Arg¹⁶⁷ of AT_1) at the top of TM4 and Lys²¹⁶ (Lys¹⁹⁹ of AT_1) and more.

Almost complete divergence between AT_1 and AT_2 receptors is seen in the third intracellular loop and more extensive differences in the carboxyl terminal tail (C-tail). The amino acid sequence of the rat AT_2 receptor indicates that it has five potential *N*-glycosylation sites with the consensus amino acid sequences -Asn-X-Ser/Thr. It is possible that carbohydrate chains would give rise to increased and diverse molecular weights of the AT_2 receptor. Southern hybridization analysis of restriction enzyme fragments of rat genomic DNA using rat AT_2 cDNA as a probe did not produce evidence for a subtype of the AT_2 receptor. Thus, the molecular weight divergence for the AT_2 receptor ranging from 60 to 140 kDa may be due to a difference in the extent of glycosylation, as shown by Servant et al. (1994).

An earlier discriminator of the AT_1 and AT_2 receptors, before the isoform-specific antagonist became available, was the reducing agent dithiothreitol (DTT). The DTT sensitive Ang II receptor turned out to be the AT_1 receptor, whereas DTT increased the ligand binding capability of the AT_2 receptor and the AT_2 receptor remained stable for hours (Chang, Loti, Keegan, 1982; Whitebread et al., 1989; Chang and Loti, 1990; Speth et al., 1991). This property was exploited for the direct purification of the AT_2 receptor protein (see below). Another

discriminator is Sar-*p*-benzoylphenylalanine⁸-angiotensin II, which can be used for selective labeling of AT_2 by a photoaffinity technique. Although it binds to the bovine adrenal AT_1 receptor with a K_d value of 6.5 nM and human myometrium with a K_d value of 0.39 nM, its covalent binding upon illumination takes place only with the AT_2 receptor (Bosse et al., 1990). The selective labeling of the AT_2 receptor is in contrast to another photoaffinity labeling peptide, Sar¹-D-azido Phe⁸-Ang II, which is specific for the AT_1 receptor (Guillemin et al., 1986). The [¹²⁵I]-radiolabeled peptide was used to visualize AT_2 receptors in various cells to determine their molecular sizes by SDS gel electrophoresis. Cells known to contain the AT_2 receptor produced radiolabeled AT_2 receptor bands of 68 kDa (human uterine myometrium), 91 kDa (R3T3 cells), and 113 kDa (PC12 cells). However, endoglycosidase treatment reduced the myometrial 68-kDa receptor to 40- and 31-kDa proteins, the 91-kDa R3T3 receptor was reduced to 46- and 31-kDa and the 113-kDa PC12 AT_2 receptor to 55 and 31 kDa. These results may involve artifacts of proteolytic digestion. More decisive evidence for glycosylation diversity came from the inhibition of *N*-glycosylation by treatment of rat PC12 cells with tunicamycin (Servant et al., 1996). The 140-kDa AT_2 receptor in untreated PC12 cells was reduced to 63- and 47-kDa glycosylated and 32-kDa unglycosylated receptors. An interesting byproduct of these studies is the observation of nonglycosylated receptor expression on the cell surface.

The structural diversity of the AT_2 receptor is still a complex and unresolved issue. Attempts have been made to purify the AT_2 receptor protein (Ciuffo et al., 1993a) by combining affinity chromatography on CGP42112-Sepharose and size separation by gel filtration and SDS gel electrophoresis from AT_2 solubilized by CHAPS from neonatal rat kidney which had a 71-kDa molecular mass. The murine neuroblastoma cell line N1E-115 cell line contains both AT_1 and AT_2 receptors. The latter increases upon cellular differentiation. Ang II receptors were solubilized from membranes by the detergent CHAPS (1%). The labile AT_1 receptor could be removed by denaturation and the AT_2 receptor was affinity purified on an Ang II-Sepharose affinity column by Siemens et al. (1991). They found that the AT_2 receptor from N1E 115 can be separated to two different forms, peak I and peak III, by a Sepharose column chromatography. Peak III seemed to be an AT_2 receptor whose ligand binding activity is enhanced by the reducing agent DTT, but the stable GTP analog, GTP-γS, did not suppress the binding. Ligand binding to a receptor under peak I was clearly reduced by GTP-γS, but treatment with DTT reduced the ligand binding function (Siemens et al., 1994b). The peak I components consisted of two proteins 110 and 60 kDa. It has been proposed that the 110 kDa is a dimer of 60 kDa that is stabilized by the agonist (Yee et al., 1994). Interestingly, stabilization of the AT_2 receptor on the cell membrane of R3T3 cells by

an as yet undefined endogenous ligand, which is not identical with Ang II, has recently been described by Csikos et al. (1998).

B. Regulation of the AT_2 Receptor

The AT_2 receptor gene is expressed ubiquitously at a very high level in the fetus and it declines precipitously after birth in many but not all tissues. In the skin, for instance, it decreases to undetectable levels, but in certain tissues such as the adrenal and heart, the decline stops at certain levels, and AT_2 receptor expression persists for the remainder of life. In uterine myometrium, the AT_2 receptor is expressed under nonpregnant conditions and declines during pregnancy, but returns to nonpregnant levels after parturition (de Gasparo et al., 1994). These few examples demonstrate clearly that the AT_2 receptor expression is regulated. Theoretically, such regulatory changes may be controlled by transcription and/or by changing the stability of mRNA.

Post-transcriptional (translational) regulation also seems to take place. After confluency, the AT_2 binding sites increase in R3T3 cells without an increase in mRNA (Dudley et al., 1991), whereas mRNA is low in the growth phase and increases in the preconfluent state. The AT_2 receptor in these cells may signal to activate protein phosphotyrosine phosphatases (PTP), which inactivates mitotic or hypertrophic growth. Teleologically, its activity should then be expressed more or less constantly in normal nongrowing cells. That this receptor lacks an internalization and desensitization mechanism is compatible with its role to maintain differentiated cells in a quiescent state (Unger, 1999).

Attempts to investigate the transcriptional regulation have begun. AT_2 genomic DNA have been cloned from mouse (Nakajima et al., 1993; Ichiki et al., 1994) and humans (Koike et al., 1994). The behavior of the AT_2 receptor gene expression in rat vascular smooth muscle cells from the spontaneously hypertensive (SHR) and Wistar Kyoto or Sprague-Dawley rats are markedly different. This finding is in line with the observation that AT_2 receptor binding in endothelial cells from SHR is markedly higher than in those from Wistar Kyoto rats (Unger, 1999). The 5'-flanking region of rat genomic DNA was also cloned to determine its functional elements (Ichiki and Inagami, 1995a).

The expression of the AT_2 receptor was shown to be dependent on growth factors or growth states. In mouse R3T3 cells, AT_2 mRNA is expressed when growth is arrested in the confluent state and is decreased in the vigorous growth phase (Dudley et al., 1991; Dudley and Summerfeldt, 1993). Prolonged serum depletion in the presence of insulin or IGF-1 stimulates the expression of AT_2 receptor in vascular smooth muscle cells, whereas serum growth factors, like PDGF, lysophosphatidic acid, and phorbol ester rapidly abolished AT_2 receptor expression (Kambayashi et al., 1993a, 1996). Similar observations with respect to IGF-1 as well as interleukin-1 β

(IL-1 β) were made in R3T3 fibroblast cells (Ichiki et al., 1995; Kambayashi et al., 1996). In contrast, growth factors (phorbol ester, lysophosphatidic acid, and basic fibroblast growth factor) markedly suppressed mouse AT_2 mRNA expression in the fibroblast cells but did not completely eliminate it as they did in vascular smooth muscle cells (Ichiki et al., 1995a,b, 1996).

The transcriptional activity of the 5'-flanking region of the mouse AT_2 receptor gene was investigated further by using constructs consisting of truncated 5'-flanking sequences (up to 1.2 kb) fused to the luciferase gene in R3T3 cells. An AP-1 site may transmit the suppressive effects of noninsulin growth factors. Thus, PKC-activating growth factors and phorbol esters rapidly down-regulate the AT_2 receptor expression (Kambayashi et al., 1993a; Kizima et al., 1996). The C/EBP site may mediate the positive transcription effect of IL-1 β as it stimulates the expression of C/EBP β and γ (Ichiki and Inagami, 1995a,b, 1996).

AT_2 receptor expression by insulin or IGF-1 is interesting in that the process has a very slow onset, taking several days. A putative insulin response sequence (IRS) is located at -126 to -117 of the 5'-flanking region of the rat AT_2 receptor gene. This sequence TAATGTTTTT is 80% homologous to the IRS in the phosphoenolpyruvate carboxylase gene TGGTGTGTTTTG (O'Brien and Granner, 1991). An interesting aspect of the effect of insulin is that the aorta of obese (fa/fa) Zucker rats, which have about 10 times higher plasma insulin concentrations than Sprague-Dawley rats, expresses AT_2 mRNA at a level clearly detectable by Northern blotting, whereas it was not detectable by the Northern blotting in the aorta of Sprague-Dawley rats (Kambayashi et al., 1996).

In these studies, an enhancer activity of the AP-1, C/EBP, NF/IL-6, IRS was not examined rigorously. Horiuchi et al. (1995) demonstrated by elegant methods that interferon regulatory factor IRF-2 attenuated AT_2 receptor expression, both in the confluent and growing cells, whereas IRF-1 enhanced AT_2 expression in confluent cells only. Several IRF binding motifs were found in the promoter in positions between -453 and -225. Whether these elements alone account for up-regulation is not yet known. Tissue-specific expression and widespread low level expression of the AT_2 receptor rather point to complex mechanisms of AT_2 receptor regulation.

C. AT_2 Receptor Diversity

Although the AT_2 receptor was cloned and purified, there remains a nagging question as to whether the AT_2 receptor is a single entity, because there is evidence for divergence in the molecular and functional properties (Siemens et al., 1994a). As discussed above, the large disparity of molecular size was attributed to a difference in the size of carbohydrate structure (Servant et al., 1994) but it could be also due to dimerization or oligomerization (Yee et al., 1994). Although DTT enhances

ligand binding of the AT_2 receptor in most tissues investigated, in some this is not the case (Tsutsumi and Saavedra, 1992b). Most intriguing is the observation that agonist binding of typical AT_1 receptors, including those that had been cloned and expressed, is not suppressed by GTP γ S, or pertussis toxin. Some AT_2 receptors in rat locus ceruleus and thalamic or geniculate nuclei are markedly affected by these reagents, which affect the heterotrimeric G proteins, G_i and G_o . On the other hand, GTP γ S has no effect on the AT_2 receptor in R3T3 cells or the inferior olive (Table 3). The lack of effect has also been seen in other cells expressing the AT_2 receptor (Tsutsumi and Saavedra, 1991). Moreover, AT_2 mRNA was not detected by *in situ* hybridization using a sequence of the cloned AT_2 receptor as an antisense oligonucleotide probe (Jöhren et al., 1996). The question as to whether diversity in the functional properties of the AT_2 receptor in different types of cells and tissues is due to another molecular species, or to additional factors associated with it, may be addressed by investigating various modes of the signal transduction mechanism of the AT_2 receptor associated with the molecular diversity.

D. Targeted AT_2 Receptor Gene Overexpression and Deletion

The use of AT_2 receptor antagonists has provided evidence for a variety of functions of the AT_2 receptor in several different types of cells and tissues including cardiovascular, renal, adrenal, central nervous, as well as dermal mesenchymal systems. Gene manipulation to increase, decrease, or abolish the expression of a gene in question are effective approaches to assess the pathophysiological roles of the gene or its product. Transgenic transfection of AT_2 receptor expression vectors into COS-7 cells (Kambayashi et al., 1993b; Mukoyama et al., 1993), and vascular smooth muscle *in vivo* (Nakajima et al., 1995) in "gain of function" approach have proven to be efficient methods. Whereas cardiac targeted overexpression of the AT_2 receptor in mice did not cause obvious morphological or functional changes, Ang II infusion paradoxically decreased blood pressure and produced a negative chronotropic effect (Masaki et al., 1998). Moreover, Tsutsumi et al. (1999) reported stimulation of bradykinin activity and nitric oxide production in AT_2 transgenic mice following inhibition of the Na⁺/H⁺ exchanger.

The targeted gene deletion (AT_2 knockout mouse or AT_2 null mouse) method produced a very interesting strain of mice (Hein et al., 1995a; Ichiki et al., 1995a). In contrast to the hypertension and impaired vascular responses observed in AT_1 -deficient mice, knockout of the AT_2 receptor leads to elevation of blood pressure and increased vascular sensitivity to Ang II (Hein et al., 1995a; Ichiki et al., 1995). This has suggested that the AT_2 receptor may exert a protective action in blood pressure regulation by counteracting AT_1 receptor function. Such an action could be exerted in part by reduced expression of the AT_1 receptor, which is increased in the vascular smooth muscle of AT_2 -deficient mice (Tanaka et al., 1999). However, the sustained hypersensitivity to Ang II in such animals is also attributable to loss of the counter-regulatory action of renal bradykinin and cyclic GMP formation (an index of NO production) (Siragy et al., 1999). The relative contributions of these two factors to AT_2 -dependent vascular regulation, and the extent to which AT_2 receptor deficiency could contribute to sustained blood pressure elevation, as in human hypertension, have yet to be determined.

The AT_2 receptor null mouse *Agtr2*(-/-) also showed behavioral changes and proclivity to have renal diseases. However, despite its massive expression in the mesenchymal tissues of skin, kidney, as well as in the brain and heart in fetal stages of development, the AT_2 receptor null mouse does not show discernible gross anatomical defects or deformity to date. The early fetal form of the kidney mesonephros is programmed to disappear during the fetal development. Its tubular tissue is densely surrounded by mesenchymal cells expressing AT_2 mRNA (Kakuchi et al., 1995). However, its disappearance also takes place in the fetus of the AT_2 receptor null mouse. The medulla of the metanephros is also filled with AT_2 receptor-expressing mesenchymal cells, which undergo apoptosis as tubulogenesis proceeds (Kakuchi et al., 1995). Again, kidney formation in the AT_2 receptor null mice takes place normally and newborn mice grew to adult mice with a normal pair of kidneys (Ichiki et al., 1995b). These studies with AT_2 receptor gene deleted mice indicate that the AT_2 receptor is not essential for apoptosis in nephrogenesis.

Functionally, the AT_2 receptor seems to exert a hypotensive effect—although this function is still discussed controversially—and to blunt the sensitivity of AT_1 receptors to Ang II. Its role in the regulation of cardiac,

TABLE 3
Binding characteristics of the AT_2 receptor*

Treatment	Atypical AT_2		AT_{2A}	
	Ventral Thalamic Nuclei	Medial Geniculate Nucleus	Locus Ceruleus	Inferior Olive
GTP γ S affect	K_D : 100% increase	Slight increase	120% increase	No increase
Pertussis toxin treatment	Agonist binding reduced to 45%	Agonist binding reduced to 63%	Agonist binding reduced to 45%	No increase

* Taken from Tsutsumi and Saavedra (1992a).

renal, and adrenal function is still under investigation. As for the renal function, the role of the AT_2 receptor as a regulator (or modulator) of pressure natriuresis is still controversial (Keiser et al., 1992; Lo et al., 1995; Siragy and Carey, 1996). Whereas studies with AT_2 receptor agonists or antagonists in intact rats suggested an antidiuretic and antinatriuretic effect of the AT_2 receptor, results in AT_2 receptor knockout mice showed the opposite effects (Siragy et al., 1999).

Although AT_2 receptor null mice did not produce gross morphological abnormalities, vascular differentiation driving the expression of the constituents of the contractile apparatus was altered. The expression of α -caldesmon and calponin are delayed in the aorta, suggesting that the AT_2 receptor enhances the differentiation of vascular smooth muscle cells and is involved in vasculogenesis (Yamada et al., 1999). In addition, more detailed studies with a larger population of AT_2 receptor null mice are producing exciting evidence that a loss of the AT_2 receptor in the AT_2 null mice results in the development of congenital urinary tract anomalies with a 23% penetrance.

1. Behavioral Changes in AT_2 Receptor Null Mice. In the brain, the AT_2 receptor is expressed in distinct areas (Obermüller et al., 1991), prominently in the locus ceruleus (Rowe et al., 1990a,b; Saavedra, 1992). The locus ceruleus contains a high concentration of noradrenergic nerve endings that release norepinephrine upon treatment with Ang II (Summers and Phillips, 1983) and sends out long noradrenergic projections to the cerebral cortex, hypothalamus, and hippocampus. The AT_2 receptor is also expressed in the central amygdaloid nucleus (Song et al., 1992), and/or medial amygdaloid nucleus (Saavedra, 1992). Loss of the AT_2 receptor in these areas could entail behavioral effects.

AT_2 receptor null mice showed a markedly reduced exploratory behavior when placed in a new environment with ambulation reduced by 40% (Hein et al., 1995a; Ichiki et al., 1995). After water deprivation, AT_2 null mice show a greater stimulation of dipsogenesis (Hein et al., 1995a).

E. Signaling Mechanisms of the AT_2 Receptor

Studies on hormone receptor signaling mechanisms are aimed at explaining cellular and physiologic responses by intracellular biochemical processes. As Nahmias and Strosberg (1995) point out, the search for major physiologic functions of the AT_2 receptor is progressing and several interesting clues are at hand. The AT_2 receptor seems to open a delayed rectifier potassium channel at least in hypothalamic neuronal tissues (Kang et al., 1994, 1995), to close a T-type Ca^{2+} channel (Buisson et al., 1992, 1995), to suppress tissue and cellular growth (Nakajima et al., 1995; Meffert et al., 1996; Munzenmaier and Green, 1996; Stoll et al., 1995; Tsuzuki et al., 1996a,b), to induce (neuronal) cell differentiation (Lafamme et al., 1996; Meffert et al.,

1996; Gallinat et al., 1997; Stroth et al., 1998; Côté et al., 1999; Gendron et al., 1999) and to support apoptosis (Yamada et al., 1996; Chamoux et al., 1999; Gallinat et al., 1999). In addition, the AT_2 receptor may exert hypotensive effects (Scheuer and Ferrone, 1993; Ichiki et al., 1995) and appears to influence behavior (Hein et al., 1995a; Ichiki et al., 1995).

The consensus among investigators working with cells expressing the AT_2 receptor exclusively without the AT_1 receptor, such as R3T3 (Dudley et al., 1990, 1991), PC12W (Bottari et al., 1991, 1992; Leung et al., 1992; Webb et al., 1992), ovarian granulosa cells (Pucell et al., 1991), and COS-7 cells expressing cloned AT_2 cDNA (Kambayashi et al., 1993b; Mukoyama et al., 1993) is that the AT_2 receptor does not modulate cytosolic Ca^{2+} or cyclic AMP, which are sensitive indicators of the heterotrimeric G $_q$ protein-coupled phospholipase C β activation and G $_i$ or G $_o$ -coupled activation or inhibition of adenylyl cyclase, respectively. Furthermore, agonist binding did not induce receptor internalization (Dudley et al., 1991; Csikos et al., 1993), nor did treatment of plasma membrane with stable GTP analogs (e.g., GTP- γ S or GDP-NH-P) result in a decrease of ligand binding. Heterotrimeric G proteins show a binding of a stable analog of GTP upon stimulation by agonist to their respective receptor. Stimulation of the AT_2 receptor in membranes from human myometrium, bovine cerebellar cortex, and rat adrenal glomerulosa did not result in an increase in the binding of [35 S]GTP- γ S (Bottari et al., 1991). These findings, obtained before the molecular structure of the AT_2 receptor had been disclosed, led the authors to propose that the AT_2 receptor was not coupled to a heterotrimeric G protein since the above are general criteria for the family of G protein-coupled receptors. Thus, even though the AT_2 receptor, as we know today, has basic structural features commonly shared by GPCR (seven transmembrane domain receptors), it did not reveal any functional features ascribable to this class of receptors.

An elaborate study on the binding of the AT_2 labeled with [125 I]-Sar 1 -Ang II indicated that the AT_2 receptor binds to G $_{12/13}$ or G $_{12}$ (Zhang and Pratt, 1996). However, the mode of interaction may be somewhat different from those seen with other G $_i$ -coupling receptors.

There is evidence that the AT_2 receptor inhibits the G $_q$ -coupled phospholipase C activation by the AT_1 receptor. Wounded rat skin expresses both AT_1 and AT_2 receptors (Kimura et al., 1992; Viswanathan and Saavedra, 1992). When rat skin slices are incubated with Ang II (10^{-7} – 10^{-4} M), the production of inositol-monophosphate is stimulated and is abolished by losartan. The AT_1 receptor-mediated inositol monophosphate production is markedly potentiated by the AT_2 antagonist PD123319 at 10^{-8} – 10^{-6} M (Gyurko et al., 1992). Although the results were obtained at high concentrations of Ang II and AT_2 antagonist, these observations indicate that

AT₂ receptor could negatively modulate the AT₁ receptor-mediated phospholipase C activation.

The effect of Ang II on cGMP in AT₂ receptor-expressing cells has been controversial (Dudley et al., 1991; Summers et al., 1991; Summers and Myers, 1991; Bottari et al., 1992; Leung et al., 1992; Webb et al., 1992; Brechler et al., 1993; Kambayashi et al., 1993b; Reagan et al., 1993b; Israel et al., 1995). The reason for this discrepancy is not yet clear. More recent *in vivo* studies on the effect of the AT₂ receptor in the kidney indicate that the stimulation of the AT₂ receptor increases renal medullary and cortical interstitial cGMP by a bradykinin-dependent mechanism (Siragy and Carey, 1996, 1997a,b; Siragy et al., 2000). Similar observations were made by Golke et al. (1998) in the aorta of stroke prone SHR infused with Ang II, and some therapeutic effects of AT₂ receptor blockade are clearly mediated by the AT₂ receptor and kinin stimulation (Liu et al., 1997). Mice overexpressing the AT₂ receptor have confirmed these findings (Tsutsumi et al., 1999). Thus, the signaling mechanisms of the AT₂ receptor have been less clearly defined as yet compared with those of the AT₁ receptor. In addition to the above-mentioned signaling pathways including G_i protein coupling and cGMP (NO) generation, activation of protein tyrosine phosphatase, serine/threonine phosphatase 2A (PP2A), protein kinase phosphatase (MKP-1) acting on both phosphotyrosine and phosphothreonine as well as SHP-1 tyrosine phosphatase have been reported (for details see Blume et al., 1999; Horiuchi et al., 1999; Millat et al., 1999).

1. Dephosphorylation and Inactivation of the Mitogen-Activated Protein Kinases ERK1 and ERK2. MAPK plays a key role in cellular proliferation, and its activation can be detected by highly sensitive methods. VSMC transfected with an AT₂-expressing vector responded to Ang II by a very slight increase in MAPK activity, whereas untransfected VSMC clearly increases MAPK. The AT₂ antagonist markedly increased the MAPK activity (both ERK1 and ERK2 isoenzymes) close to the level attained in untransfected VSMC when stimulated with 10^{-7} M Ang II. These results indicate that stimulation of the AT₂ receptor by Ang II suppresses the AT₁ receptor-mediated activation of MAPK (Nakajima et al., 1995). Although adult aortic media do not seem to express AT₂ at measurable levels, the carotid artery may express it to a limited degree (Viswanathan et al., 1991). AT₂ receptor expressed, even in a limited quantity, at the edge of neointima, (or, for that matter, in a healing wound) may suppress MAPK in the growing edge. The serine/threonine phosphatase, PP2A, which is activated by Ang II stimulation of the AT₂ receptor in the neonatal hypothalamic neurons, was recently shown to inactivate MAPK following the stimulation of the AT₁ receptor. In the presence of the AT₂ receptor antagonist, PD123319, MAPK activity was markedly enhanced. Thus, the AT₂ receptor is able to block MAPK activation either by the dephosphorylation of serine/threonine phosphate by

PP2A or tyrosine phosphate by PTPs, most likely by MAPK phosphatase, MKP-1 (Huang et al., 1996).

The influence of the AT₂ receptor on MAPK activity may also depend on the biological program engaged by the receptor under defined experimental conditions. Thus in PC12W cells expressing AT₂, but not AT₁, receptors, treatment with Ang II under conditions allowing for cell differentiation induced a short-lasting increase in ERK1 and ERK2 activity as was seen with NGF, a classical differentiation factor. Costimulation with NGF and Ang II led to initial MAPK stimulation but the subsequent NGF-induced plateau of ERK1 and ERK2 stimulation was suppressed by the AT₂ receptor (Stroth et al., 2000). Altered ERK activity was also observed in the heart of AT₂ transgenic mice suggesting that the ERK inactivated by the AT₂ receptor has a physiological role *in vivo* (Masaki et al., 1998).

Further evidence for a G_i-coupled activation of PTP was obtained in vascular smooth muscle cells in culture, even if the AT₂ receptor was not expressed in these cells. Instead of using Ang II to stimulate G_i, it was activated by a 22 amino acid residue peptide with the sequence of the third intracellular loop of the AT₂ receptor protein, which was transferred into the cells expressing the AT₂ receptor by LipofectAMINE liposome. Cells which received the AT₂-related peptide showed small but significant attenuation of serum-stimulated MAPK and thymidine incorporation. The suppression of thymidine incorporation was reversed by pretreatment of cells with pertussis toxin, which indicates the role of a G_i or G_o protein. The inhibition of MAPK activation by serum was also reversed by sodium orthovanadate, a tyrosine phosphatase inhibitor but not by okadaic acid, a serine/threonine phosphatase inhibitor, which indicates that the third intracellular loop of AT₂ receptor activates a PTP via a G_i or G_o protein (Hayashida et al., 1996). In the neuroblastoma cell line, N1E-115, the AT₂ receptor has been found to activate the catalytic activity of SH-PTP1, a soluble PTP implicated in termination of signaling by cytokine and growth factor receptors, resulting in ERK inactivation (Bedecs et al., 1997).

Recently, ceramide, which is linked to phosphatase activation, was proposed to be the second messenger in AT₂ receptor-mediated apoptosis, (Gallinat et al., 1999; Lehtonen et al., 1999), and pertussis toxin and orthovanadate blocked its production. A negative cross-talk appears to exist between AT₂ and AT₁ receptors not only on a functional level as has been observed by various authors (see Unger, 1999) but also on the level of intracellular signaling. For instance, stimulation by Ang II, interferon, EGF, or PDGF of rat adult vascular smooth muscle cells transfected with AT₂ receptor cDNA inhibits AT₁ receptor-mediated ERK activation and tyrosine phosphorylation of STAT without influence on Janus kinase (Horiuchi et al., 1999). However, these results also indicate that the G_i-coupled activation of protein tyrosine phosphatase(s) or protein serine/threo-

nine phosphatase is not targeted narrowly to specific channel proteins and that many other proteins can be their substrates.

2. Activation of Phospholipase A_2 and Prostacyclin Generation. In contrast to cells in which the AT_2 receptor is the predominant or exclusive Ang II receptor, cardiac ventricular myocytes are interesting as they express both AT_1 and AT_2 receptors (Busche et al., 2000). In the hypertrophic ventricles of SHR and two-kidney one-clip hypertensive rats, AT_1 receptor expression is increased along with AT_2 receptor density (Suzuki et al., 1993). Thus, the heart offers unique materials for studies on interactive regulation of AT_1 and AT_2 receptors. The AT_2 receptor mediates sustained arachidonic acid release in isolated pure cardiac myocytes, and this effect is completely blocked by the AT_2 -specific antagonist PD123317. Losartan suppresses it by about 50%. On the other hand, the AT_1 receptor supports largely the release of inositol phosphates. The fact that 1 mM DTT does not completely abolish arachidonic acid release, but completely eliminates inositol phosphate, supports the role of the AT_2 receptor in the activation of phospholipase A_2 but not phospholipase C (Lokuta et al., 1994).

Kohout and Rogers (1995) found evidence indicating that the AT_2 receptor-mediated release of arachidonic acid may contribute to the activation of Na^+/HCO^- symporter system (NBC), which increases the pH value of myocytes by 0.08 pH unit. This stimulation of the symporter system by Ang II can be blocked by the AT_2 blocker PD123319, but not by the AT_1 blocker losartan. Furthermore, superfusion of myocytes with exogenous arachidonic acid (5 μ M) mimicked the Ang II-mediated alkalinization. Thus, the arachidonate release and symporter activation is a unique function of the AT_2 receptor in the heart. These observations were extended by Sandmann et al. (1998) in an *ex vivo* study in rats after myocardial infarction. Whereas pretreatment with an ACE inhibitor prevented the postinfarct up-regulation of both ion transporter systems, the Na^+/H^+ -exchanger (NHE-1) and NBC, in cardiac tissue, the AT_1 receptor antagonist, valsartan, selectively blocked the increase in NHE-1 and the AT_2 antagonist, PD123319, selectively blocked the increase of the NBC.

AT_2 receptor-dependent production of prostacyclin (PGI_2) was reported in differentiated adipocytes Ob1771 in culture. The PGI_2 formation is seen only in differentiated Ob1771 cells, and PGI_2 thus formed induces differentiation of undifferentiated Ob1771 cells in coculture by a paracrine mechanism. This action is blocked by PD123177, but not by losartan, indicating that the prostacyclin formation is mediated by the AT_2 receptor (Daimont et al., 1994). The mechanism involved in this signaling pathway remains to be clarified.

In summary, diverse AT_2 receptor signaling pathways were unveiled, which include activation of serine/threonine phosphatase PP2A and subsequent opening of the delayed rectifier K^+ -channel, activation of cytosolic

PTPs, which may lead to closing of the T-type Ca^{2+} channel, inactivation, but in some cases also transient activation, of MAPK, the inhibitory effect presumably through PP2A, MKP-1, or other PTP, and the activation of phospholipase A_2 (Nouet and Nahmias, 2000). With respect to the specific pathways or substrates involved in AT_2 receptor-mediated intracellular signaling, there are still numerous unresolved problems such as GTP- γ S sensitivity, PTP activation, or cGMP production.

F. Tissue Distribution of the AT_2 Receptor

To identify the physiologic functions of the AT_2 receptor, investigations were initiated on tissue-specific expression, changes in AT_2 expression in relation to ontogenic stages and tissue development, and identification of cell types *in vivo* and in cell lines carrying the AT_2 receptor. In these studies, the use of isoform selective antagonists for the AT_1 and AT_2 receptors enabled investigators to detect biphasic binding of Ang II, which indicated the coexistence of AT_1 and AT_2 receptors. Later, cloning of AT_2 cDNA allowed for specific identification of AT_2 mRNA expressed in various tissues (Kakuchi et al., 1995; Shanmugam et al., 1995; Jöhren et al., 1996). Distribution of the AT_2 receptor appears to be tissue- and species-specific. Both AT_1 and AT_2 receptors are expressed in the adrenal gland at varying ratios in different regions. In the rat adrenal medulla, the ratio of AT_1 to AT_2 receptors was approximately 20:80 (Chang and Lotti, 1990), whereas in rat, rabbit, monkey and human adrenal cortex, the AT_2 receptor comprised ~10 to 40% of the total angiotensin binding sites (Chiu et al., 1989a; Whitebread et al., 1989; Chang and Lotti, 1990). Other tissues that expressed the AT_2 receptor at a high proportion, as compared to the AT_1 receptor, were the nonpregnant human uterus (Whitebread et al., 1989; Criscione et al., 1990; Bottari et al., 1991; de Gasparo and Levens, 1994); sheep uterine myometrium (Cox et al., 1993); bovine cerebellar cortex (Bottari et al., 1991); and rat ovarian follicular granulosa cells (Pucell et al., 1991). Species dependence exists, as only 40% and 60% of Ang II binding sites are AT_1 in rat and rabbit uterus, respectively (Dudley et al., 1990; Bottari et al., 1991). The proportion of the AT_2 receptor in the kidney cortex is less than 10% of the AT_1 receptor in the rat and rabbit, and ~55% in monkey (Chang and Lotti, 1991). On the other hand, the heart in the rat, rabbit, and monkey contains the AT_2 receptor in small but finite and measurable amounts, which comprises about 30% of the total Ang II binding sites (Chang and Lotti, 1991). The rat and primate pancreas were found to contain AT_1 and AT_2 receptors (Chappell et al., 1992, 1994). Expression of the AT_2 receptor was particularly high in pancreatic acinar cells (Chappell et al., 1995). Table 4 summarizes adult and fetal tissues that express AT_2 receptors.

Some fetal tissues express the AT_2 receptor at high levels. As summarized in Table 4, in many of these tissues the AT_2 receptor emerges on embryonic days 11

to 13 (E11-E13) and reaches a maximal level on E19. The AT_2 receptor then rapidly declines in newborn animals to lower levels or to undetectable levels. As examined by autoradiographic techniques and/or in situ hybridization, expression of the AT_2 receptor was particularly dense in differentiated mesenchymes, such as mesenchymal tissues in the tongue, subdermal, and s.c. regions of the skin and the diaphragm. In these tissues, the preponderant fetal Ang II binding sites are AT_2 (>97%) (Grady et al., 1991; Feuillan et al., 1993; Grady and Kalinyak, 1993; Shanmugam et al., 1995). The skin and tongue are the tissues in which fetal expression of AT_2 receptor is particularly intense, however, rapid postpartum disappearance is seen. In many other tissues where fetal AT_2 receptor expression level is detectable, a precipitous lowering of the AT_2 receptor is observed. The very dense expression of Ang II receptors in rat fetal skin cells noted by Millan et al. (1989) seems to be AT_2 receptor (Feuillan et al., 1993). These cells were isolated and cultured. Earlier passage cells express more AT_2 than AT_1 receptor. Switching of AT_2 to AT_1 expression, or AT_2 -expressing cells to AT_1 -expressing cells seems to take place (Johnson and Aguilera, 1991). In some tissues, AT_2 receptors reach undetectable levels (submucosal cells of the stomach and intestine or trachea). On the other hand, in certain tissues the process of rapid postpartum decrease is arrested, and the AT_2 receptor remains at detectable levels in the adrenal medulla, zona glomerulosa, (Shanmugam et al., 1995), pancreas (Chappell et al., 1994), uterus (Cox et al., 1993), and heart (see below).

In the fetal kidney, AT_2 receptor expression is seen mostly in the mesenchymal cells of differentiating cortex and medulla, which are surrounding glomeruli in the cortex and tubular tissues in the medulla (Kakuchi et al., 1995). The mesenchymal cells will undergo apoptosis and will be replaced by tubular tissues. The role of the

AT_2 receptor in fetal development is not yet clearly established. Although the apoptosis of the AT_2 receptor-expressing cells in fetal tissue development is an attractive concept, hemizygotic male AT_2 receptor gene deleted mice (the AT_2 receptor gene is localized to the X-chromosome) and homozygotic female AT_2 receptor gene null mice undergo normal fetal development, and newborns and adults do not show any abnormality in the gross morphology of the skin, tongue, kidney, or adrenal. A delay in vasculogenesis was observed, however (Yamada et al., 1998).

Although in many tissues the AT_2 receptor undergoes a unidirectional decrease in its expression, this receptor also undergoes reversible changes. The myometria of the human, sheep, and rat express measurable AT_2 receptors at a high level (Crisicione et al., 1990; Bottari et al., 1991; Cox et al., 1993; de Gasparo et al., 1994). In sheep uterus, for instance, the ratio of AT_1 : AT_2 receptor is 15:85. In the pregnant ewe, the AT_2 receptor is reduced by more than 90% and AT_1 is reduced by 60% with the AT_1 to AT_2 ratio being reversed to 80:20. However, upon parturition, the AT_2 receptor rapidly returns to the high prepartum levels (Cox et al., 1993). Similar observation was made in human myometrium during pregnancy (de Gasparo et al., 1994). The AT_2 receptor also emerges during wound healing of the skin (Kimura et al., 1992; Viswanathan and Saavedra, 1992). This increase occurs particularly in the superficial dermis. A low level of AT_2 receptor expression was also seen in the neointimal tissue of rat carotid artery following balloon catheterization (Janiak et al., 1992; Viswanathan et al., 1994b). These changes in the uterus during pregnancy or in wound healing in the skin and brain indicate that the AT_2 receptor has a definitive but not yet identified regulatory function in these tissues.

I. Brain. Although circumventricular organs of the brain respond to circulating Ang II, an endogenous brain

TABLE 4
Tissue distribution of the AT_2 receptor and ontogenic change

Tissues	Fetus	Newborn	3 wk	8 wk	Reference
Adrenal					Shanmugam et al., 1995
Cortex	+	+	+	+	
Medulla		+	+	+	Feuillan et al., 1993
Ovary					
Follicular granulosa	+	+		+	Fucell et al., 1991
Kidney					
Cortex	+++				Shanmugam et al., 1995
Medulla (outer strip)	+	++			Shanmugam et al., 1995; Kakuchi et al., 1995
Heart	±				Shanmugam et al., 1995
Uterus					
Myometrium				+	
Blood vessels					
Heart	±				Shanmugam et al., 1995
Aorta	+			+	Feuillan et al., 1997; Shanmugam et al., 1995
Pancreas	+				Chappell et al., 1992
Trachea	+				Shanmugam et al., 1995
Stomach	+				Shanmugam et al., 1995
Mesenchyme	+				
Skin	+++	±			Grady et al., 1991; Shanmugam et al., 1995; Feuillan et al., 1993
Tongue	+++	±			Grady et al., 1991; Feuillan et al., 1993
Skeletal muscle	+				Feuillan et al., 1993

renin-angiotensin system can generate Ang II in tissues protected from circulating Ang II by the blood-brain barrier. Ang II generated by this system reacts with angiotensin receptors within the brain. The brain angiotensin receptors have been studied and reviewed extensively by Gehlert et al. (1991a,b), Rowe et al. (1992), Saavedra (1992), Song et al. (1992), and Höhle et al. (1995). An expression of the AT_2 receptor in fetal (E18) brain tissues was reported in several areas: inferior olive, paratrigeminal nucleus, and hypoglossal nucleus. Saavedra (1992) determined AT_1 and AT_2 receptor expression in various regions of the brain, and its age dependence in 2- and 8-week-old rat brains. Although AT_1 receptor expression did not show marked age dependence, the AT_2 receptor showed a significant decrease from 2 to 8 weeks of age (see Table 5).

Obermüller et al. (1991) investigated the distribution of Ang II receptor types in adult rat brain nuclei using competitive radioligand binding. Whereas midbrain and brain stem contained AT_1 and AT_2 receptors in comparable concentrations, AT_1 receptors were by far predominant in several hypothalamic nuclei, although a limited amount of AT_2 receptors was detectable in most of them. Although minor differences may exist among these studies, they share the following observations: AT_2 receptor expression is consistently high in the cerebellar nuclei, inferior olive, and locus ceruleus in the brain stem,

which is rich in noradrenergic neurons. In contrast to the AT_1 receptor, which participates in various central cardiovascular functions and is expressed in the hypothalamus and in brain stem nuclei (nucleus of the solitary tract, dorsal motor nucleus of vagus at a low level), the AT_2 receptor is much less present in distinct hypothalamic and brain stem nuclei associated with the regulation of cardiovascular functions. The presence of the AT_2 receptor in the dorsal motor nucleus of vagus is not completely settled.

Although the distribution of AT_2 receptors in the brain is well known, their effects are still not clear. As outlined above, brain AT_2 receptors may play a role in cognitive functions and certain types of behavior such as exploration or drinking (Hein et al., 1995a). On the other hand, they may also antagonize the central effects of angiotensin peptides in osmoregulation mediated via AT_1 receptors (Höhle et al., 1995, 1996). A possibly important role of AT_2 receptors in neuroregeneration and neuroprotection will be dealt with below.

2. Heart. Earlier studies suggested that myocytes of rabbit and rat hearts contain AT_1 and AT_2 receptors in comparable quantities as shown in Table 6 (Rogg et al., 1990; Scott et al., 1992; Sechi et al., 1992b). However, questions remained as to the cell-specific localization of these receptors *in situ*. A recent study using single cell RT-PCR has demonstrated that in the adult rat about 50% of cardiomyocytes contain the AT_1 receptor, whereas the AT_2 receptor is much more scarce with only about 10% carrying this receptor type (Busche et al., 2000).

Autoradiographic studies of the rat heart show about a 2-fold increase in AT_1 and AT_2 receptors after birth, and their receptor numbers are about equal from E16 through 10 to 16 weeks of age (Sechi et al., 1992b). They are expressed in the myocardium of all four chambers, as well as in the vascular smooth muscles of the aorta and pulmonary arteries. However, very little ligand binding was seen in the coronary artery. The conduction system (the atrioventricular and sino-aortic nodes) was reported to contain both AT_1 and AT_2 receptors by Sechi et al. (1992b), whereas Saavedra et al. (1993), Brink et al. (1996), and Wharton et al. (1998), showed only AT_1 receptors in these tissues.

Primary culture of cardiomyocytes from neonatal rat left ventricles showed about a 50% decrease in the AT_2

TABLE 5
Distribution of the AT_2 receptor in the brain—effect of age

	2-wk-old	8-wk-old
	fmol/mg protein (mean \pm S.E.M.)	
Regions containing only the AT_2 receptor		
Persistent AT_2 receptor with age		
Lateral septal nucleus	59 \pm 6	18 \pm 3
Ventral thalamic nuclei	101 \pm 8	24 \pm 8
Mediodorsal thalamic nucleus	165 \pm 11	38 \pm 13
Locus ceruleus	289 \pm 19	98 \pm 13
Principal sensory trigeminal nucleus	75 \pm 6	15 \pm 4
Parasolitary nucleus	230 \pm 15	57 \pm 14
Inferior olive	1328 \pm 61	181 \pm 32
Medial amygdaloid nucleus	159 \pm 8	94 \pm 9
Medial geniculate nucleus	338 \pm 24	71 \pm 6
Transient expression of the AT_2 receptor		
Anterior pretectal nucleus	53 \pm 8	N.D.
Nucleus of the optic tract	101 \pm 13	N.D.
Ventral tegmental area	101 \pm 11	N.D.
Posteroventral tegmental nucleus	110 \pm 21	N.D.
Hypoglossal nucleus	141 \pm 11	N.D.
Central medial and paracentral thalamic nucleus	202 \pm 14	N.D.
Lateralodorsal thalamic nucleus	110 \pm 10	N.D.
Oculomotor nucleus	98 \pm 13	N.D.
Regions containing both AT_1 and AT_2 receptors		
Persistent AT_2 receptor with age		
Superior colliculus	145 \pm 5	65 \pm 8
Cingulate cortex	19 \pm 4	8 \pm 4
Transient expression of the AT_2 receptor		
Cerebellar cortex	59 \pm 6	N.D.

N.D., not determined.

TABLE 6
Coexistence of AT_1 and AT_2 in cardiac tissues

	% of AT_1 + AT_2		References
	AT_1	AT_2	
Rabbit ventricle	60	40	Rogg et al., 1990
Rat (10-wk-old)	65	50	Sechi et al., 1992b
Myocytes	65	95	Matsubara et al., 1994
Fibroblasts	>95	<5	Villareal et al., 1993
Fibroblasts (adult)	100		
Bovine	67	33	Nozaki et al., 1994
Human	33	69	Regitz-Zagrosek, 1995

receptor from those of E19 fetuses, whereas the AT₁ receptor did not decrease (Matsubara et al., 1994). In contrast, the AT₂ receptor in cultured fibroblasts collected from 1-day-old rat heart was less than 10% of the AT₂ receptor in fibroblasts cultured from E19 fetal heart, whereas there was no change in AT₁ receptor number. In cardiac fibroblasts cultured from 7-day-old rats, AT₂ receptor density was negligible and the majority of the Ang II receptor population was reported to be AT₁, whereas in cultured cardiomyocytes AT₂ receptor numbers are maintained at a finite and measurable level (about 50% of AT₁). Bovine and human ventricular and atrial myocardium also contain both AT₂ and AT₁ receptors (Rogg et al., 1991; Nozawa et al., 1994; Regitz-Zagrosek et al., 1995). Although the AT₂ receptor is usually expressed at low density in adult, it is up-regulated to different extents in pathological circumstances such as cardiac hypertrophy, myocardial infarction, cardiomyopathy, and congestive heart failure (Matsubara, 1998; Unger, 1999). Interestingly, both in nonfailing and explanted end-stage human heart the AT₂ receptor population measured in a binding assay (65% of total Ang II receptor) is greater than AT₁, and there may be a correlation between the density of the AT₂ receptors and the severity of heart failure (Rogg et al., 1996). Wharton et al. (1998) also observed a significant increased density of high-affinity binding sites in endocardial, interstitial, perivascular, and infarcted regions of the ventricle of patients with end-stage ischemic heart disease or dilated cardiomyopathy, greater than in adjacent noninfarcted myocardium. The border zone between noninfarcted and infarcted myocardium was rich in microvessels with perivascular AT₂ receptors. Ohkubo et al. (1997) reported that in the heart of cardiomyopathic hamster (Bio 146), both AT₁ and AT₂ receptors were increased during heart failure (153% and 72%, respectively). In human, the expression of the AT₂ receptor was markedly (3-fold) increased in patients with dilated cardiomyopathy at both protein and mRNA levels compared with patients with acute or well organized old myocardial infarction (Tsutsumi et al., 1998). In contrast, the AT₁ receptor expression was significantly down-regulated. The AT₂ receptor sites were highly localized in the interstitial region in the fibrotic areas where fibroblasts are present. Collagen and fibronectin formation from fibroblasts were suppressed by the AT₁ antagonist TCV116, and increased by the inhibition of AT₂ receptor with PD123181 in the cardiomyopathic hamster. These results are intriguing as they suggest the possibility that the AT₁ and AT₂ receptors modulate extracellular matrix formation in an opposite way as has already been suggested from experiments using cardiac microvascular endothelial cells (Fischer et al., 1996).

Although much remains to be learned about the various functions of cardiac AT₂ receptors, their distribution and time- and event-driven regulation suggest roles

in cardiac development, repair and remodeling with actions opposing those mediated by cardiac AT₁ receptors.

3. Kidney. The kidney is a major target organ of Ang II. Ang II exerts its regulatory function on both hemodynamic and tubular functions, and most of its actions seem to be explained by AT₁ receptor functions. The AT₁ receptors are indeed abundantly expressed in small cortical arteries, glomeruli, proximal tubules, and interstitium. The AT₂ receptor is, however, also expressed in the kidney. Ontogenic studies on rat and humans by autoradiography, in the presence of losartan (Sechi et al., 1992a; Ciuffo et al., 1993b), on rat (Shanmugan et al., 1995), and on mouse (Kakuchi et al., 1995) showed marked development-dependent changes in AT₂ receptor expression in the kidney.

In the mouse (Kakuchi et al., 1995), on embryonic days 12 to 16 (E12-E16) AT₂ mRNA was densely expressed in the mesenchymal cells of the mesonephros surrounding the mesonephric tubules. In this developmental period, the mesonephros begins regression, and a possible function of the AT₂ receptor in the apoptotic loss of mesonephros can be envisioned. On E14, the AT₂ receptor emerges in the interstitial mesenchymes of the kidney, but not in the glomeruli or the S-body, whereas the AT₁ receptor is richly expressed in the preglomeruli and S-bodies. On E16, AT₂ mRNA is seen in the renal capsule and inner medulla where it is prominent along the papillary duct and between the collecting ducts. AT₂ receptor on the medullary ray extend into the cortex, whereas the detection signals are reduced to undetectable levels after birth (Kakuchi et al., 1995; Shanmugan et al., 1995).

About 10 to 15% of the renal Ang II binding sites were blocked by CGP42112 (de Gasparo et al., 1990; Zhuo et al., 1992, 1993, 1998). The outermost layer of the cortex outside of the glomeruli and inner layer of medulla seem to show signaling for the AT₂ receptor, the proximal convoluted tubule is a candidate site for AT₂ receptor localization, which is functional in the renal tubular system, as its existence has been reported by Dulin et al. (1994). The same group also reported the presence of the AT₂ receptor in cultured rat mesangial cells by binding studies (Ernsberger et al., 1992). However, quantitative autoradiographic studies of rat renal glomeruli by Ciuffo et al. (1993b) did not detect the AT₂ receptor in rat renal glomeruli. Reviewing many of these studies, it appears that the competitive ligand binding autoradiography or *in situ* hybridization does not provide sufficient sensitivity for detecting a low-level expression of AT₂, comprising only 10 to 20% of total binding. Along this line, earlier studies by a similar technique by Gibson et al. (1991) did not detect AT₂ receptor in the rat renal cortex. By contrast, in rhesus monkey kidneys, AT₂ receptors (PD12198 sensitive sites) were seen in the juxtaglomerular apparatus and in arterial smooth muscle cells.

Ozono et al. (1997) examined the expression of the AT₂ receptor in the fetal, newborn, and adult kidney by im-

munohistochemistry using antibodies raised against the peptide epitope with the amino acid sequence of the N terminus of the rat AT_2 receptor. Positive immunohistochemical staining of the AT_2 receptor was observed in the mesenchymal cells and ureteral bands of the 14-day-old fetal kidney and in the glomeruli, tubules, and vessels in the 19-day-old fetal and newborn kidney. Glomeruli expressing the AT_2 receptor were localized mainly in the outer layers of the renal cortex. In the young and adult rat, AT_2 receptors were present in glomeruli at a substantially diminished level. Lowering dietary sodium intake increased glomerular and interstitial AT_2 receptors. In human kidney, the AT_2 receptor is clearly present in large preglomerular vessels of the renal cortex (Grone et al., 1992) and in the tubular interstitium (Chansel et al., 1992; Goldfarb et al., 1994). Possible renal functions of the AT_2 receptor have recently been reviewed (Carey et al., 2000).

4. Vasculature. Although it is well known that the AT_1 receptor, present in vascular smooth muscle, mediates the contractile and hypertrophic effects of Ang II, the presence of the AT_2 receptor in the vasculature *in vivo* was seen only by autoradiography. Its exact site of localization and functional roles are yet to be clarified. In coronary endothelial cells derived from SHR, AT_2 receptor mRNA as well as AT_2 binding were present with a ratio AT_1/AT_2 of about 80%/20% (Stoll et al., 1995). In ontogenic studies on AT_2 receptor expression in fetal (E18), 2-week-, and 8-week-old rat aorta autoradiographic determination of specific binding of [125 I]-Sar¹-Ang II in the presence of losartan and PD123177 was performed (Viswanathan et al., 1991). Interestingly, the AT_2 receptor was the major or almost exclusive Ang II receptor in the fetal aorta (E18), accounting for 85% of the total binding. Even at 2 weeks of age the AT_2 was still the dominant form but it became a minor Ang II receptor at 8 weeks of age (25%). However, the total Ang II receptor sites decrease after birth and throughout the process of maturation from a very high level (300 fmol/mg of protein) in the fetus down to less than 10 fmol/mg of protein in 8 weeks. Autoradiography showed the AT_2 receptor in the medial layer even at 8 weeks of age. Similar results were obtained by competitive binding of the vascular membrane fraction (Chang and Lotti, 1991). This small but finite population of AT_2 receptors in the media of the vasculature may explain a rapid pressor and subsequent depressor effect of Ang III infused into rats pretreated with losartan. The AT_2 blocker almost completely eliminated the depressor phase (Scheuer and Perrone, 1993). The presence of AT_2 receptors even at a low level may be compatible with the increased sensitivity to the pressor action of Ang II at a low concentration (10^{-12} – 10^{-10} M) under AT_2 blockade. Thus, it has been speculated that the AT_2 blockade unmasks the sensitivity of the vascular AT_1 receptor to a low concentration of Ang II (Hong et al., 1994).

Formation of neointima following balloon catheterization of the rat carotid artery can be suppressed by angiotensin converting enzyme inhibitors or AT_1 antagonists (see above). However, there was a report that an AT_2 agonist was efficient in preventing neointima formation, but not an AT_1 antagonist (Janiak et al., 1992). Autoradiographic studies of Ang II receptor expression indicated a marked increase in AT_1 and a decrease in AT_2 receptors in the balloon catheter-treated aorta, and equally elevated AT_1 but no expression of AT_2 receptors in the normal and injured carotid artery (Viswanathan et al., 1994b). However, the neointimal area of the vessels transfected with and expressing the AT_2 receptor transgene was decreased by 70% compared to untransfected or control-vector transfected vessels. This inhibitory effect on the development of the neointima was blocked by an AT_2 receptor antagonist, PD123319 (Nakajima et al., 1995). The *in vivo* localization of the AT_2 receptor in the aorta of rats may provide a basis for a possible interpretation of the interesting report that chronic blockade of AT_2 rather than AT_1 receptor antagonizes Ang II-induced aortic hypertrophy and fibrosis (Levy et al., 1996). In a model of chronic Ang II infusion, Cao et al. (1999) reported on an increased mesenteric weight and wall-lumen ratio of the vessels as well as proliferation of smooth muscle cells. These vascular changes were attenuated by both AT_1 and AT_2 receptor antagonists. Moreover, AT_1 receptor blockade was associated with down-regulation of both receptors, whereas AT_2 receptor blockade was associated with reduced Ang II binding to only the AT_2 receptor. Although these observations suggest that vasotrophic effects of Ang II could, at least partially and under certain experimental conditions, be mediated by the AT_2 receptor type, this topic is still controversially discussed. The marked AT_2 -mediated increase of aortic cGMP under Ang II infusion in SHR as observed by Gohlke et al. (1998) is just one example speaking against AT_2 as a vasotrophic factor.

Despite the fact that the media of rat thoracic aorta express the AT_2 receptor, smooth muscle cells derived from the aorta and cultured *in vitro* do not (Kambayashi et al., 1996; Ichiki et al., 1996). This is possibly due to down-regulation of the AT_2 receptor gene by growth factors in serum-containing culture medium since AT_2 receptor expression can be regained upon prolonged serum depletion in the presence of insulin (Kambayashi et al., 1996).

Coronary endothelial cells cultured from adult SHR contain AT_1 and AT_2 receptors in a ratio of about 80/20% as determined by the demonstration of AT_2 mRNA, AT_2 binding and functional studies (Stoll et al., 1995). The same authors showed that in these cells, serum- or basic fibroblast growth factor-induced proliferation is dose-dependently inhibited by Ang II (Metsärinne et al., 1992; Stoll et al., 1995) and that this antiproliferative effect is sensitive to AT_2 receptor blockade. Furthermore, that under nonstimulated conditions, the mito-

genic activity of Ang II is suppressed by the AT₁ antagonist losartan but is markedly enhanced by the AT₂ antagonist PD123177 (Stoll et al., 1995). This is presumably because the mitogenic action of AT₁ receptor is partially suppressed by the AT₂ receptor. The antiproliferative effect of the AT₂ receptor has subsequently been confirmed in several laboratories and also extended to nonvascular cell systems (Meffert et al., 1996; Munzenmaier et al., 1999; Goto et al., 1997; Van Kesteren et al., 1997; Maric et al., 1998).

5. *Pancreas, Lung, Thymus, and Other Tissues.* Canine and primate pancreas were found to contain the AT₂ receptor at a relatively high level (Chappell et al., 1992, 1994). This receptor was characterized by high binding affinity ($K_d \sim 0.48$ nM) for Ang II, a 2-fold enhancement of binding by DTT and inhibition of radioactive Ang II binding by AT₂-specific antagonists. Of the total Ang II binding sites approximately 70% seems to be AT₂. Autoradiographic studies showed a predominance of AT₂ over AT₁ receptors throughout the pancreas including islet cells, acinar and duct cells, as well as vasculature cells (Chappell et al., 1991, 1992). Subsequently, the rat pancreatic acinar cell line AR42J was shown to contain a high level of AT₂ receptors (250 fmol/mg of protein) with concomitant AT₁ receptors at ~ 10 to 15% of the level of the AT₂ receptor (Chappell et al., 1995). The pancreas also contains a large amount of angiotensinogen and its mRNA studied (Chappell et al., 1991).

The distribution of the AT₁ and AT₂ receptor in human lung was studied using immunohistochemistry with specific polyclonal antibodies and with *in situ* hybridization. The AT₁ receptor mRNA and protein were localized on vascular smooth muscle cells, macrophages, and, in particular, in the stroma underlying the airway epithelium. In contrast, the AT₂ receptor mRNA and protein was observed in the epithelium, with strong staining on the bronchial epithelial cell brush border and also on many of the underlying mucous glands. The AT₂ receptor was also present on some endothelial cells (Bullock et al., 1999). The thymus was shown to contain sites able to bind Sar¹-Ang II at a high affinity. This binding is inhibited by an AT₂ antagonist (Correa et al., 1994).

There are tissues or cells that are devoid of AT₂ receptor when using either ¹²⁵I-Ang II in the presence of AT₁ selective antagonists or the AT₂-specific binding agent ¹²⁵I-CGP 42112. These tissues are the liver (Whitebread et al., 1989; Dudley et al., 1990) and the pituitary (Leung et al., 1991).

6. *Cells in Primary Culture and Cell Lines Expressing the AT₂ Receptor.* To clarify the signaling mechanism and pathophysiological significance of the AT₂ receptor, numerous types of cells derived from tissues known to contain the AT₂ receptor were prepared or identified. They provided useful materials for studies on AT₂ recep-

tor signaling, pathophysiological roles, and regulation for cloning and purification.

Among primary cultured cells were neuronal cells dispersed from rat neonatal hypothalamus (Summers and Myers, 1991), neonatal rat cardiomyocytes (Lokuta et al., 1994; Suzuki et al., 1993), adult rat aortic endothelial cells (Stoll et al., 1995), rat fetal skin cells (Tsutsumi et al., 1991), and rat fetal fibroblasts (Johnson and Aguilera, 1991).

Rat adrenomedullary pheochromocytoma PC12 cells express both AT₁ and AT₂ receptors reflecting their adrenal medullary origin where both AT₁ and AT₂ receptors are coexpressed. A subline, PC12W, which expresses a large amount of AT₂ but not AT₁ receptors (Speth and Kim, 1990), were extensively used for studies of AT₂ signaling mechanisms and for AT₂ cloning. A subline of the Swiss mouse 3T3 fibroblast cell line, R3T3, also expresses AT₂ without AT₁ receptor (Dudley et al., 1991; Csikos et al., 1998). Although in both of these cell lines expression of the AT₂ receptor is suppressed in the cellular growth phase, AT₂ mRNA increases as they approach the subconfluent or confluent phase. When R3T3 cells are shifted to a quiescent condition by serum deprivation, the AT₂ receptor protein production increases without proportional increase in mRNA (Dudley and Summerfeldt, 1993).

An undifferentiated mouse neuroblastoma cell line, NG108-15, was shown to express the AT₂ but not the AT₁ receptor (Buisson et al., 1992) and was used in studies of the signaling mechanism: AT₂ receptor activation by Ang II resulted in phosphotyrosine phosphatase activation and closing of calcium T-channel (Buisson et al., 1995).

Another mouse neuroblastoma cell, N1E-115, expresses both AT₁ and AT₂ receptors (Reagan et al., 1990). Since AT₁ receptors can be rapidly destroyed by dithiothreitol, AT₂ receptors could be preserved in fractions solubilized by the detergent CHAPS and were used as a source for purification of the AT₂ receptor. The transformed rat pancreatic acinar cell line, AR42J, contains both AT₁ and AT₂ receptors and shows a marked rise in Ca²⁺ characteristic of the AT₁ receptor response (Chappell et al., 1995). Thus, this cell line may not be very convenient for the study of the role of AT₂ receptor. However, AR42J cells have an exocrine function that may or may not be regulated by AT₂. Ovarian granulosa cells isolated to a high degree of purity and maintained in primary culture were also very useful to study AT₂ receptor-dependent differentiation and apoptosis (Pucell et al., 1991; Tanaka et al., 1995). The cloned mouse preadipocyte cell line, Ob1771, expresses the AT₂ receptor upon differentiation in a serum-free medium and responds to Ang II by producing prostacyclin, which promotes the differentiation of the preadipocytes by a paracrine mechanism (Darimont et al., 1994). Rat VSMC in culture do not express the AT₂ receptor under regular culture conditions in the presence of fetal calf

serum (Stoll et al., 1995; Ichiki et al., 1996; Kambayashi et al., 1996), but, as outlined above, the AT_2 receptor can emerge in rat thoracic VSMC when cultured in serum-depleted medium supplemented with insulin or other insulin-like growth factors such as IGF (Ichiki et al., 1996; Kambayashi et al., 1996). Thus rat VSMC grown and maintained under ordinary culture conditions do not seem to provide convenient materials for the study of the role of the AT_2 receptor. However, VSMC can be transfected with an artificial AT_2 receptor gene consisting of an AT_2 receptor coding region with a myosin heavy chain promoter (Nakajima et al., 1995).

G. Pathophysiological Aspects of AT_2 Receptor Activation

The widespread distribution of AT_2 receptors in various brain nuclei, heart, vascular tissues, adrenal, kidney, skin, and during wound healing suggests a physiological role for the AT_2 receptor. The identification of several AT_2 receptor signaling pathways as reviewed in preceding sections implies diverse pathophysiological consequences. Studies to identify the role of the AT_2 receptor in pathophysiology have been conducted using diverse approaches, which include the use of cultured cells, in vivo vascular tissues injured by balloon catheter, transgenic expression of AT_2 receptor, targeted AT_2 receptor gene deletion (gene knockout), and chronic administration of AT_2 antagonists and agonists (Horiuchi et al., 1998; Matsubara, 1998; Unger, 1999; Carey et al., 2000).

1. *The AT_2 Receptor Can Induce Apoptosis.* The effect of the AT_2 receptor does not stop at the inhibition of cellular proliferation but is associated with cellular programs such as differentiation and regeneration (see below) and, under appropriate conditions, also with apoptosis.

In PC12W cells, expressing only AT_2 but not AT_1 receptors, nuclear condensation, fragmentation, and marginalization were observed in serum-free medium containing NGF (1 ng/ml) after treatment with 10^{-7} M Ang II for 2 days (Yamada et al., 1996; Gallinat et al., 1999). Characteristic internucleosomal DNA fragmentation was also observed. In R3T3 cells, serum depletion seemed to be the major contributor to apoptotic DNA fragmentation although activation of AT_2 receptor has an additional effect.

Skin fibroblasts collected from mice embryos with genetic deletion of the AT_2 receptor did not show any DNA fragmentation characteristic of apoptosis after stimulation with Ang II, whereas fibroblasts from wild mice did (Li et al., 1998b). Ovarian granulosa cells undergo apoptosis during follicular atresia. These cells contain the AT_2 but not the AT_1 receptor (Pucell et al., 1991). Tanaka et al. (1995) showed that these cells in culture underwent apoptotic DNA fragmentation when depleted of follicle-stimulating hormone. Although no direct effect of added Ang II on the apoptosis was observed, the AT_2

receptor level in these cells was increased by the removal of follicle-stimulating hormone and the addition of Ang II, indirectly suggesting a possible role of the AT_2 receptor in apoptosis and even perhaps in ovulation.

"Programmed cell death" is an important concept in developmental morphogenesis. The fetal kidney expresses the AT_1 receptor in the preglomerular S-bodies, whereas the AT_2 receptor is expressed in fetal renal mesenchymal cells, which are replaced by tubular tissues in the later stages of renal development. Mesonephros also express the AT_2 receptor in the mesenchyme surrounding its tubular system. Mesonephros are known to disappear by apoptosis (Kakuchi et al., 1995). However, the nephrogenesis occurs even in AT_2 receptor null mice. Thus, triggering of the AT_2 receptor-mediated activation of MKP-1 and apoptosis may be contingent on additional growth suppressive measures such as serum depletion.

The mechanism behind AT_2 -mediated apoptosis appears complex as both AT_1 and AT_2 receptors are involved in the process (de Gasparo and Siragy, 1999). The calcium flow between endoplasmic reticulum and mitochondria and the ratio *Bcl2/Bax*, which is modulated by both AT_1 and AT_2 receptors play a key role in homeostasis between cell growth and cell death (Kajstura et al., 1997; Berridge et al., 1998; Fortunato et al., 1998; Tea et al., 1998). Horiuchi et al. (1997b) reported that AT_2 receptor stimulation in PC12W cells dephosphorylates *Bcl2* by activation of MKP-1. In PC12W cells, AT_2 stimulation markedly lowered MAPK. This effect was suppressed by pretreatment with vanadate and pertussis toxin, indicating that a G protein-driven PTP decreased MAPK activity. The PTP was shown to be MKP-1, since the elimination of MKP-1 by an antisense oligonucleotide transfection abolished DNA fragmentation (Yamada et al., 1996). Activation of caspase may be involved in AT_2 receptor-induced apoptosis in umbilical venous endothelial cells (Dimmeler et al., 1997). A stimulation of ceramide, which replaces the JNK-stress-activated protein kinase pathway leads to caspase stimulation (Hayashida et al., 1996).

Gallinat et al. (1999) observed that in PC12W cells, kept under conditions allowing for apoptosis, the AT_2 receptor-induced apoptosis was associated with an Ang II concentration-dependent ceramide production. Because sphingomyelin concentrations were unaltered by Ang II these findings suggested a *de novo* synthesis of ceramide by AT_2 receptor stimulation. This report was subsequently confirmed by Lehtonen et al. (1999).

Stretch also activates apoptosis following increased expression of the transcriptional factor p53 and up-regulation of the local renin-angiotensin system, whereas *Bcl2* expression is decreased. p53 indeed binds to the promoter of the angiotensinogen and the AT_1 receptor genes and stimulates production of Ang II and expression of the AT_1 receptor (Pierzchalski et al., 1997; Leri et al., 1998).

Together, these reports clearly indicate that the AT_2 receptor is able to induce apoptosis, although the signaling pathways await further investigation. It should be noted, however, that the proapoptotic features of the AT_2 receptor can only be unveiled under specific experimental conditions, for instance serum deprivation/NGF dependence, which generally prepare the grounds for apoptosis.

2. Effects on Vascular Tone. The presence of AT_2 receptors in the vasculature has been discussed above. Whereas the vascular antiproliferative and neointima-reducing effects of AT_2 receptor stimulation are now well established (Horiuchi et al., 1998; Unger, 1999), the participation of the AT_2 receptor in the regulation of vascular tone is still controversial. When a bolus injection of Ang III was given i.v. into anesthetized rats, both pressor and depressor effects were observed. Under AT_1 receptor blockade, Ang III exerted a dose-dependent depressor effect, which was abolished when both AT_1 and AT_2 receptors were blocked (Scheuer and Perrone, 1993). In rabbit abdominal aorta, the AT_2 blocker PD123177 unmasked losartan-sensitive pressor effects of Ang II at a low-concentration range of 10^{-12} to 10^{-10} M where the peptide alone was not pressor. These *in vivo* results suggested that a pressor action of the AT_1 receptor was masked by the AT_2 receptor (Hong et al., 1994). AT_2 receptor gene null mice prepared by Ichiki et al. (1995) show an elevated systolic and diastolic pressure, indicating that the AT_2 receptor has a depressor effect. In a renal wrap Grollman hypertension model, AT_1 receptor blockade normalized blood pressure, whereas AT_2 receptor antagonism significantly increased blood pressure but decreased renal interstitial concentration of bradykinin and nitric oxide (Siragy and Carey, 1999). These results suggest that the AT_2 receptor mediates counterregulatory vasodilation. Similarly, AT_2 receptor activation with CGP42112 facilitates in SHR the depressor response caused by an AT_1 receptor antagonist (Barber et al., 1999). On the other hand, in adult conscious SHR a concomitant infusion of Ang II and the AT_2 antagonist, PD123319, did not result in blood pressure elevation, whereas the AT_1 antagonist, losartan, consistently lowered blood pressure in the presence or absence of Ang II (Gohlke et al., 1998).

Thus, although the results of several studies suggest that the AT_2 receptor exerts depressor effects upon stimulation by Ang II or III, there is also evidence against the idea of AT_2 as a vasodilator. It appears that, under normal conditions, the pressor effect of the AT_1 receptor exerts a dominant effect, and overall effect of Ang II is directed to blood pressure elevation. A defect of the AT_2 receptor, either genetic or otherwise, could lead to an elevation in blood pressure or increased sensitivity to Ang II or III. On the other hand, blockade of AT_1 receptor in juxtaglomerular cells by selective AT_1 receptor antagonists increases circulating Ang II, which will then interact with the unopposed AT_2 receptor. Stimulation

of AT_2 receptors could thus contribute to a further decrease of blood pressure. However, the exact mechanism of AT_2 receptor-mediated depressor effect is still not clear.

3. Vascular Hypertrophy and Fibrosis and the AT_2 Receptor. Arterial hypertrophy, remodeling, and fibrotic changes induced by chronic administration of Ang II have been believed to be due to diverse effects mediated by the AT_1 receptor. However, when normotensive rats received a chronic (3 weeks) s.c. infusion of Ang II (120 ng/kg/min), Ang II + losartan (10 mg/kg/day) or Ang + PD123319, it was the AT_1 receptor blocked (with losartan) rat that showed a marked increase in collagen and elastin in the thoracic aorta, despite normal blood pressure maintained by losartan. By contrast, collagen and elastin in the Ang II-PD123319-treated rats were at low control levels despite markedly elevated blood pressure. Media thickness was also increased in the AT_2 -blocked rats rather than AT_1 -blocked animals (Levy et al., 1996; Cao et al., 1999). A persuasive explanation for these intriguing observations, which were not confirmed by Li et al. (1998a) and are in disagreement with a host of reports on the antiproliferative effects of the AT_2 receptor in many tissues including the vasculature (Horiuchi et al., 1999; Unger, 1999) is not yet available.

4. Renal Tubular Function. The presence of an AT_2 -like (termed AT_{1B}) receptor in renal tubules particularly in the proximal tubules have been reported by Douglas and associates (Douglas, 1987; Ernsberger et al., 1992; Dulin et al., 1994). However, AT_2 mRNA is not readily detected in adult rat kidney. On the other hand, the AT_1 receptor is clearly expressed in the glomeruli and in the inner stripe of the outer medulla. Glomerular AT_1 receptors may control the hemodynamic function of the kidney.

The AT_1 receptor seems to have some detectable effect on renal tubular function. The AT_2 selective antagonist, PD123319, infused i.v. at 300 μ g/kg/min into anesthetized dogs increased free water clearance 4-fold and sodium excretion 3-fold, whereas the AT_1 selective losartan had only insignificant effects on the tubular functions (Keiser et al., 1992). In these experiments, renal blood flow was decreased only by 10%. On the other hand, salt replete dogs infused with PD123177 into the renal artery did not show appreciable changes in free water clearance and natriuresis (Clark et al., 1993). When the hemodynamic function and tubular function are dissociated by maintaining the renal blood flow constant using inflatable aortic cuffs above and below the renal artery of an unilaterally nephrectomized rat (Roman et al., 1984), infusion of the AT_2 blocker, PD123319, rapidly induces an increase in diuresis and natriuresis. As the extent of diuresis was perfusion pressure-dependent, it is considered to represent "pressure natriuresis". CGP42112, an AT_2 agonist, suppresses the diuretic response. The glomerular filtration rate remained remarkably constant (Lo et al., 1995). These results can be

interpreted to mean that AT_2 receptor stimulation by Ang II induces Na^+ retention when kidney perfusion pressure is increased. In contrast, in conscious rats with a chronic microdialysis cannula in the renal medullary interstitium, AT_1 receptor blockade showed a marked diuretic effect, whereas AT_2 receptor blockade by PD123319 did not exert any actions on water clearance or natriuresis. When animals were placed on a low-salt diet, cGMP and PGE_2 in the microdialysate from the kidney interstitial fluid were generally increased 2- to 3-fold over a 5-day period. Upon infusion of losartan, PGE_2 was reduced to the basal level of rats on regular diet, whereas cGMP was not affected. PD123319 markedly increased (~4-fold) PGE_2 and reduced cGMP to basal levels. Losartan plus PD123319 reduced PGE_2 to basal levels overriding the effect of PD123319, whereas cGMP was essentially regulated by PD123319. These results indicate that the AT_2 blocker, PD123319, rapidly induces an increase in diuresis and production of cGMP, which is regulated primarily by the AT_2 receptor, whereas the AT_1 receptor has no effect. On the other hand, PGE_2 production was abolished by losartan and was markedly increased by PD123319 (Siragy and Carey, 1996, 1997a,b). Similar microdialysis has indicated that tissue bradykinin, nitric oxide, and $PGF_{2\alpha}$ formation were released upon stimulation of the AT_2 receptor (Siragy et al., 2000).

In AT_2 receptor knockout mice, a suppressor dose of Ang II inhibits natriuresis and diuresis suggesting that the AT_2 receptor stimulation physiologically increase pressure natriuresis and that this effect is sustained over a prolonged period (Siragy et al., 1999). An explanation for the discrepancy with the data of Lo et al. (1995) is not obvious as yet.

5. Neuronal Cell Differentiation and Nerve Regeneration. Cultured Schwann cells express both AT_1 and AT_2 receptors, and Ang II decreases the expression of the neurite-promoting protease nexin-1. Blockade of the AT_1 receptor or stimulation of the AT_2 receptor leads to a severalfold increase of nexin-1 favoring nerve regeneration (Bleuel et al., 1995). Similarly, treatment of non-differentiated NG108-15 or PC12W cells with Ang II or nerve growth factor induces growth arrest and morphological differentiation of neuronal cells including neurite outgrowth and up-regulation of polymerized tubulin as well as microtubule-associated protein MAP2c expression and differential regulation of neurofilament M (Lafamme et al., 1996; Meffert et al., 1996; Gallinat et al., 1997; Stroth et al., 1998). These effects were mimicked by the AT_2 receptor agonist, CGP42112A, and abolished by coinubation with the antagonist, PD123319. The underlying signaling mechanisms may include inhibition of $p21^{ras}$ as well as activation of MAPK (Gendron et al., 1999; Stroth et al., 2000). Repression of c-fos and c-jun expression does not seem to be involved in AT_2 receptor-mediated growth arrest and cell differentiation in PC12W cells (Steckelings et al.,

1998). Moreover, there is a marked up-regulation of AT_2 receptor mRNA in regenerating neurons in response to nerve crush coinciding, as an AT_2 expression wave from proximal to distal, with the regeneration of nerve fibers (Gallinat et al., 1998). Both in vitro and in vivo, Ang II promotes axonal elongation of postnatal retinal explants and dorsal root ganglia neurons and, in adult rats, axonal regeneration of retinal ganglion cells after optic nerve crush. This effect is completely abolished by an AT_2 receptor antagonist but not affected by an AT_1 receptor antagonist (Lucius et al., 1998). Thus, the AT_2 receptor plays a role in neuronal cell differentiation and nerve regeneration via regulation of the cytoskeleton, whereas the AT_1 receptor may inhibit this process (Unger, 1999).

H. Summary

AT_2 receptor expression is widespread. However, in several tissues it disappears after birth or when cells are transferred in culture in the presence of serum and growth factor. On the other hand, a sometimes dramatic up-regulation of the AT_2 receptor can occur after tissue injury. Research on the AT_2 receptor has revealed actions differing greatly from those of the AT_1 receptor such as antiproliferative effects in several tissues, cellular differentiation, nerve regeneration, and apoptosis. It appears that the AT_2 receptor often plays the role of a modulator of biological programs in tissue development or repair.

The signaling mechanisms of the AT_2 receptor are diverse, and only a few of them have as yet been characterized reasonably well. In some cases they are coupled to G_i proteins. One pathway in neurons (and perhaps other tissues) involves activation of protein serine/threonine phosphatase PP2A, which leads to the activation of the delayed rectifier K^+ -channel (outward K^+ current in neuronal cells). This will result in hyperpolarization of plasma membranes that suppress cellular activities stimulated by depolarization. A second signaling pathway involves the activation of protein phosphotyrosine phosphatases (PTPases), which are of pivotal importance to prevent or rapidly shut off undesired and uncontrolled growth of normal tissues.

The AT_2 receptor lacks the mechanism of rapid desensitization, internalization, and low-affinity shift by GTP. Molecular mechanisms underlying such a unique signaling mechanism have not been clarified and warrant further intensive investigation.

Arachidonic acid release from cardiac myocytes leading to the activation of Na^+/HCO_3^- symporter may be a unique regulatory mechanism of cardiac myocytes. Release of nitric oxide with subsequent formation of cGMP appears to be another important intracellular effect of AT_2 stimulation occurring in vascular and renal tissues. In many adult tissues, the modulatory or suppressive actions of the AT_2 receptor, often counteracting the AT_1 receptor-mediated stimulatory actions, require measure

of reduction in activities, which is technically more difficult than measurement of increased activity from a very low level. This may have been the reason for which the AT_1 receptor has sometimes escaped the attention of investigators. Chronic application of AT_2 antagonists and AT_2 receptor gene deletion are beginning to reveal the role of AT_2 receptor in the maintenance of, or restoring normality not only in the cardiovascular system but also in other tissues e.g., the central nervous system. A possible role of AT_2 receptor in apoptosis of mesenchymal cells in conjunction with other factors is beginning to be clarified as an important basis of organogenesis, failure of which may lead to some childhood diseases.

IV. The AT_4 Receptor

In 1990, during the process of purifying and sequencing the AT_1 receptor type, it was noticed that heat-denatured purified receptor from the bovine adrenal gland lost binding with ^{125}I -Sar¹, Ile⁶Ang II, whereas ^{125}I -Ang III binding persisted. At first it was suspected that an angiotensin receptor specific to Ang III was isolated. However, with sufficient peptidase inhibitors added to prevent the conversion of Ang III to shorter fragments, this binding activity was lost. This was puzzling given that the two known receptor types at that time, AT_1 and AT_2 , each accepted Ang II and Ang III as ligands, albeit with different affinities. A fragment of Ang III was suspected to be acting at this new site because Sar¹, Ang II, Sar¹, Ile⁶Ang II (Sarile), Sar¹, Ala⁶Ang II (Saralasin), DuP753 (losartan), PD123177, CGP42112A, Ang II(1-7) and Ang III did not serve as ligands (Harding et al., 1992; Swanson et al., 1992). In fact, ^{125}I -Ang II(3-8)[Ang IV] did bind at this site reversibly, saturably, and with high affinity ($K_d = 1$ nM) (Harding et al., 1992; Swanson et al., 1992; Sardinia et al., 1993). Having identified a ligand that bound at this site it was possible to determine its brain distribution using *in vitro* autoradiographic techniques. The brain distribution of this receptor site was unlike that of either the AT_1 or AT_2 receptors. The greatest concentrations of what has now been termed the AT_4 site (IUPHAR Nomenclature Committee, de Gasparo et al., 1995) were in structures classically associated with cognitive processes and sensory and motor functions, not in structures associated with the functions of body water balance, cardiovascular regulation, and control of reproductive hormones and behaviors where the AT_1 receptor is predominant. The following sections summarize what is presently known about this new Ang IV/ AT_4 system with emphasis on signaling mechanisms, tissue distributions, the development of analogs that act as agonists and antagonists, and the physiologies and behaviors associated with this AT_4 site.

A. Signaling Mechanisms

Given that small peptides are able to activate the AT_4 receptor and that the vast majority of small peptide

receptors are G protein-linked, it would seem logical to predict that the AT_4 receptor might be a serpentine, G protein-linked receptor as well. This, however, does not appear to be the case since the AT_4 receptor exhibits a molecular weight between 160 and 190 kDa as determined by reduced SDS-polyacrylamide gel electrophoresis. Of particular interest is the observation that the adrenal AT_4 receptor may be multimeric. This suggestion comes as a result of nonreducing gels that indicate a second specifically labeled band at 225 kDa. A similar molecular weight has been observed for other bovine tissues including heart, thymus, kidney, bladder, aorta, and hippocampus (Zhang et al., 1999). Bernier et al. (1995) have reported a similar molecular weight for the binding subunit of the AT_4 receptor in bovine aortic endothelial cells. Together these data all but preclude the linkage of AT_4 receptors to G proteins. The lack of linkage to G proteins is further supported by the observation that GTP γ S fails to alter ^{125}I -Ang IV binding in rabbit heart (Hanesworth et al., 1993), guinea pig brain (Miller-Wing et al., 1993), and rat vascular smooth muscle (Hall et al., 1993). However, a single report by Dulin et al. (1995) indicates that GTP γ S can inhibit binding in opossum kidney cells. As experienced with the AT_2 receptor, a definite conclusion has to wait the cloning of the AT_4 receptor.

The initial events that characterize AT_4 receptor intracellular signaling mechanisms are presently unknown. Nevertheless, downstream targets appear to include immediate early genes. Intracerebroventricular infusion of Ang IV in rats induces c-Fos expression in brain regions associated with cognition (Roberts et al., 1995). Angiotensin IV agonists can also stimulate c-Fos, c-Jun, and *egr-1* in isolated, nonstimulated rabbit hearts (Slinker, personal communication). Studies carried out in the laboratory of Vaughan (Kerins et al., 1995) indicate that AT_4 activation increases the expression of plasminogen activator inhibitor, PAI-1, and is blocked by coapplication of an AT_4 receptor antagonist. However, a possible involvement of the AT_2 receptor has been postulated both *in vitro* and *in vivo* (Brown et al., 1999; Chabielka et al., 1999; Goodfield et al., 1999; Sironi et al., 1999).

A final area of investigation indirectly associated with AT_4 signaling is the identification of endogenous ligands for the AT_4 receptor. The first putative ligand identified for the AT_4 receptor was the hexapeptide, Ang IV. Although Ang IV is known to be present in the circulation (Semple et al., 1976) and is generated from Ang II or Ang III (Abbold and Harding, 1988), recent studies (Møller et al., 1997) indicate that other peptides like LVV-hormophilin-7 are also capable of binding and activating AT_4 receptors. This is not surprising given that the structural requirements for AT_4 ligands are fairly minimal. Presumably, other putative ligands will be found in the future.

B. Tissue Distribution of the AT₄ Receptor

1. *Brain.* To date, brain distributions of the AT₄ binding site using in vitro autoradiography have been completed in rat (Roberts et al., 1995), guinea pig (Miller-Wing et al., 1993), *macaca fascicularis* (Møller et al., 1996a), rhesus monkey (Wright et al., 1995), and human (hippocampus only; Harding, unpublished observations), and there is cross-species consistency. The predominant brain distribution of AT₄ receptor is presented in Table 6 by comparison with the AT₁ and AT₂ receptors. The highest densities of the AT₄ site are located in regions involved in cognitive processing, and motor and sensory functions. Specifically, the AT₄ receptor site is prominent in structures associated with the cholinergic system (Møller et al., 1996a). This system is composed of two major pathways: one from the basal forebrain with cell bodies located in the nucleus basalis magnocellularis, which project primarily to the neocortex, whereas the other originates in the medial septum-diagonal band of Broca complex and projects primarily to the hippocampus (Wenk et al., 1980; Mesulam et al., 1983; Dutar et al., 1995). There are also additional projections to the amygdala and thalamus presumed to be involved in the integration of subcortical contributions to this system (Goldman and Cote, 1991), and the piriform cortex.

Equally impressive binding has been reported in structures associated with motor function including the ventral horn of the spinal cord (i.e., spinal motor nuclei) (Møller et al., 1995, 1996a; Wright and Harding, 1995) inferior olivary nucleus, motor trigeminal nucleus, vestibular and reticular nuclei of the hindbrain, red nucleus, oculomotor nucleus, substantia nigra, and ventral tegmentum of the midbrain. In the forebrain, considerable binding is present in the globus pallidus, caudate-putamen, and nucleus accumbens (Miller-Wing et al., 1993; Møller et al., 1995, 1996a). There are also high densities of AT₄ receptors in the granular cell layer of the cerebellum and deep cerebellar nuclei (Miller-Wing et al., 1993), as well as Betz cells of the primary motor neocortex (Møller et al., 1996a). AT₄ receptors have also been identified in brain autonomic nuclei such as the dorsal motor nucleus of the vagus, nucleus ambiguus, rostral ventral lateral medulla, and paraventricular nucleus of the hypothalamus (Møller et al., 1995, 1996a).

Finally, less dense distributions of AT₄ sites have been identified in sensory associated structures including spinal trigeminal nuclei, the colliculi, gracile and cuneate nuclei, and lateral geniculate nuclei, thalamic nuclei (anterior, lateral, and ventral), lateral olfactory tract, and primary sensory neocortex (Miller-Wing et al., 1993; Møller et al., 1995, 1996a).

A comparison of the adult brain structures most densely distributed with AT₁, AT₂, and AT₄ receptors reveal some overlap (Table 7). Most notably in the dorsal motor nucleus of the vagus, inferior olivary nucleus,

cerebellum, superior colliculus, lateral geniculate nucleus, and paraventricular nucleus (Höhle et al., 1995; Wright and Harding, 1995, 1997; Møller et al., 1996a). However, there are structures rather uniquely distributed with AT₄ receptors. These include: reticular formation (motor areas), motor trigeminal and vestibular nuclei, cuneate and gracile nuclei, ventral tegmental area, periaqueductal gray, caudate-putamen, medial habenula, nucleus basalis of Meynert, hippocampus, piriform cortex, Betz cells in neocortex, and granular layer of the cerebellum.

2. *Peripheral Tissue.* Table 8 provides the binding constants for AT₄ receptors located in several peripheral tissues. Bovine adrenal cortex revealed a mean (\pm S.D.) K_d value of 0.7 (\pm 0.14) nM and a B_{max} value of 3.82 (\pm 1.12) pmol/mg of protein for [¹²⁵I]-Ang IV (Harding et al., 1994). The binding of [¹²⁵I]-Ang IV at this AT₄ site could not be inhibited with Ang II, Sar¹, Ile⁶-Ang II, Ang II(1-7), DuP753, CGP42112A, PD123177. The metabolically resistant form of Ang III, [D-Arg¹]-Ang III had a significantly lower apparent binding affinity for this site.

Monkey kidney displayed a K_d value of 1.5 (\pm 0.31) nM and a B_{max} value of approximately 1.0 (\pm 0.21) pmol/mg of protein (Harding et al., 1994). Autoradiographic analyses of rat kidney indicated heavy concentrations of AT₄ receptors in the outer stripe of the medulla and a few receptors in glomeruli and the core of the medulla (Harding et al., 1994; Coleman et al., 1998). The reverse pattern was observed for [¹²⁵I]-Sar¹, Ile⁶-Ang II binding to the AT₁ and AT₂ receptor sites.

Rat, guinea pig, and rabbit hearts revealed heavy concentrations of AT₄ receptors with K_d values of 3.3 (\pm 1.1), 1.33 (\pm 0.02), and 1.75 (\pm 0.5) nM, respectively. Corresponding B_{max} values were: 0.32 (\pm 0.03), 0.14 (\pm 0.02), and 0.73 (\pm 0.16) pmol/mg of protein (Wright et al., 1995). Autoradiographic analyses indicated heavy [¹²⁵I]-Ang IV binding throughout the heart muscle. Guinea pig and bovine vascular smooth muscle evidenced considerable binding with K_d values of 0.40 (\pm 0.09) and 1.85 (\pm 0.45) nM, respectively. The B_{max} values were: 1.04 (\pm 0.24) and 0.96 (\pm 0.1) pmol/mg of protein, respectively. Binding constants for other tissues of interest, such as guinea pig colon and spleen, human bladder and prostate, are also included in Table 8.

C. Development of Agonists and Antagonists

1. *Binding Requirements of AT₄ Receptor.* The characteristics of the Ang IV molecule that result in high-affinity binding at the AT₄ type are much different than those described above for the AT₁ receptor. Specifically, they include the following. 1) Removal of the N-terminal valine significantly reduces binding affinity as compared with Ang IV ($K_d \approx 2.6 \cdot 10^{-9}$ M) (reviewed in Wright et al., 1995). 2) Glycine substitution in positions 1, 2, or 3 of the Ang IV molecule or the use of D-isomers at these positions, greatly reduces affinity (Sardinia et al., 1993). 3) On the other hand, positions 4, 5, and 6 can accom-

TABLE 7
Predominant brain distributions of the three angiotensin receptor types identified in the mammalian brain^a

	AT ₁	AT ₂	AT ₃
Structures:	Amygdala Anterior pituitary gland Area postrema Dorsal motor nucleus of the vagus Habenula Inferior olivary nucleus Interpeduncular nucleus Lateral geniculate Lateral olfactory tract Locus coeruleus Medial preoptic area Median eminence Nucleus of the solitary tract Olfactory bulb organum Vasculum of the lateral terminalis Paraventricular nucleus Septum Subfornical organ Superior colliculus Supraoptic nucleus Ventroanterior hypothalamic nucleus Red nucleus	Amygdala Caudate putamen Globus pallidus Hypoglossal nucleus Inferior colliculus Inferior olivary nucleus Lateral habenula Locus coeruleus Medial geniculate pons Reticular formation Septum Thalamus Ventral tegmental area	Amygdala Anterior pituitary gland Caudate-putamen Cerebellum Cerebral neocortex Dorsal motor nucleus of the vagus Globus pallidus Habenula Hippocampus Inferior colliculus Inferior olivary nucleus Lateral geniculate Lateral olfactory tract Locus coeruleus Nucleus basalis of magnocellularis Nucleus accumbens Nucleus cuneatus Nucleus gracilis Paraventricular nucleus of hypothalamus Periaqueductal gray piriform cortex Septum Spinal cord-ventral horn Substantia nigra Superior colliculus Thalamus Ventral tegmental area

^a Adapted from Wright and Harding (1997).

TABLE 8
Binding constants for AT₂ receptors in various species and tissues

Species	Tissue	K_d nM	B_{max} fmol/mg protein
Rabbit	Heart	1.75 ± 0.57^a	751 ± 163
Rat	Heart	3.3 ± 1.1	320 ± 66
Bovine	Adrenals	0.74 ± 0.14	3820 ± 1120
Guinea pig	Heart	1.33 ± 0.02	144 ± 19
Guinea pig	Hippocampus	1.29 ± 0.18	499 ± 62
Bovine	Vascular smooth muscle	1.85 ± 0.45	960 ± 100
Bovine	Vascular smooth muscle	0.70 ± 0.10	476 ± 87
Guinea pig	Vascular smooth muscle	0.40 ± 0.09	1040 ± 239
Monkey	Kidney	1.5 ± 0.31	1000 ± 212
Guinea pig	Colon	0.84 ± 0.08	659 ± 75
Human	Prostate	0.60 ± 0.15	1899 ± 182

^a Mean \pm S.D., $n = 2-4$. Study performed with [¹²⁵I]-Ang IV.

modate several amino acid constituents without significantly impacting affinity. A detailed study of position 1 of Ang IV revealed the following specific characteristics.

1) High affinity binding necessitates the presence of a primary amine in position 1. Methylation, cyclization, or removal of the N-terminal amine yields a dramatic loss of affinity. 2) Hydrophobic residues in position 1 produce the greatest binding affinity. Thus, norleucine¹-Ang IV with a 4-carbon straight chain aliphatic group in position 1 results in a very high affinity ($K_d \approx 3.2 \text{ nM}$, 10^{-12} M). The addition of the amino group in position 1 significantly lowered affinity as compared with analogous straight chain analogs. 3) The amino acids substituted in position 1 must be in the L-configuration. For example, replacing L-norleucine with D-norleucine in position 1 significantly reduces affinity ($K_d \approx 6 \cdot 10^{-7}$ M). Reasonably high affinity can be achieved with a CH₃-NH isostere substituted for the 1-2 bond. These isosteres permit increased rotation around the peptide bond (Sar-

dina et al., 1994). They also introduce a formal positive charge with a bond length similar to the normal peptide bond accompanied by increased metabolic stability. Position 2 of the Ang IV molecule requires an aromatic amino acid in the L-configuration to achieve high-affinity binding. Position 3 requires a hydrophobic amino acid also in the L-configuration. Thus, the requirements for significant occupancy and activation of the AT₂ receptor type include a cluster group of Val-Tyr-Ile-R₁-R₂-R₃. Removal of phenylalanine from the C terminus of Ang IV yields a binding affinity, which is nearly equivalent with that of native angiotensin IV, but is physiologically inactive as measured by its influence on blood flow. With further successive removal of amino acids, binding affinity declines such that the K_d value for Ang IV(1-4) $\approx 5.7 \cdot 10^{-8}$ M, and for Ang IV(1-3) $\approx 4.8 \cdot 10^{-7}$ M (reviewed in Wright et al., 1995). In sum, the minimal ligand requirements for an Ang IV-like peptide to bind with reasonably high affinity at the AT₂ receptor type appears to be Val-Tyr-Ile-R₁-R₂, although an agonist appears to require the addition of phenylalanine at the C terminus yielding a required structure of Val-Tyr-Ile-R₁-R₂-Phe.

2. *Antagonists of the AT₂ Receptor.* Antagonists of the AT₂ receptor type have been reasonably difficult to design and synthesize. Harding's laboratory has had success with the substitution of Val for Ile in the third position of Ang IV accompanied by reduced peptide bonds between Val-Tyr and Tyr-Val (i.e., Val-Tyr-Val-His-Pro-Phe). This compound called divalinal-Ang IV (Krebs et al., 1996) binds at the AT₂ receptor type with high affinity to a similar number of binding sites as [¹²⁵I]-Ang IV. It does not bind at the AT₁ and AT₃ receptor

types and has been shown to prevent i.c.v. Ang IV-induced c-Fos expression in rats (Roberts et al., 1995), Ang IV-induced elevation in cerebral (Kramár et al., 1997) and kidney cortical blood flow (Coleman et al., 1998), and Ang IV-induced inhibition of proximal tubule Na^+ transport (Handa et al., 1998).

D. Physiology Associated with the AT_4 Receptor

1. *Regulation of Blood Flow.* Haberl et al. (1991) have reported dilation of rabbit pial arterioles with topical application of Ang IV using the closed cranial window technique (Haberl, 1994). Ang IV-induced dilation was dependent upon pretreatment with exogenous L-arginine. Application of L-arginine alone resulted in only minimal vasodilation ($+4\%$ at 10^{-5} M), whereas coapplication of L-arginine and Ang IV produced a 20% vasodilation. Pretreatment with methylene blue blocks vasodilation suggesting that it is dependent upon an endothelial-dependent factor, probably nitric oxide (Riedel et al., 1995). A degradation product of Ang II could therefore induce this endothelium-dependent dilation (Haberl et al., 1991).

Laser Doppler flowmetry was used to measure Ang IV-induced increase in blood flow within the cerebral cortex (Kramár et al., 1997) and kidney cortex (Swanson et al., 1992; Harding et al., 1994; Coleman et al., 1998). Angiotensin IV was intra-arterially administered via the internal carotid or renal arteries, respectively. Angiotensin II induced decreases in blood flow in these preparations. Pretreatment with DuP753 blocked subsequent Ang II-induced reductions in cerebral or renal cortical blood flow, whereas both PD123177 and divalinal-Ang IV failed to inhibit these responses. In contrast, pretreatment with the antagonist divalinal-Ang IV blocked the Ang IV-induced elevation in cerebral or renal cortical blood flow, whereas pretreatment with DuP753 or PD123177 failed to influence this response. In a related study, Kramár et al. (1998) have noted that pretreatment with the NO synthase inhibitor, N^{ω} -nitro-L-arginine (L-NAME) blocked the vasodilatory effect of Ang IV suggesting that this Ang IV-induced elevation in cerebral blood flow is dependent upon the synthesis and release of NO from vascular endothelial cells. Similar results have been obtained by Coleman et al. (1998) concerning kidney cortical blood flow.

Finally, Näveri et al. (1994a,b) have noted that i.v. administration of Ang IV reversed the decreases in cerebral blood flow that accompanies experimentally induced subarachnoid hemorrhage. Pretreatment with the nonspecific AT_1 and AT_2 receptor antagonist, $\text{Sar}^1\text{-Ile}^8$ Ang II, failed to influence this Ang IV-induced effect suggesting that the elevation in blood flow was mediated by the AT_4 receptor type. Taken together these results argue in favor of important roles for angiotensins II, III, and IV in the control of blood flow. In contrast with the above findings, Gardiner et al. (1993) have noted Ang IV-induced reductions in renal and mesenteric blood

flows and vascular conductances in conscious rats using implanted pulsed Doppler flow probes. Pretreatment with DuP753 blocked these responses suggesting mediation by the AT_1 receptor type. The authors concluded that Ang IV acts as a weak agonist at the AT_1 receptor type. In agreement with these findings, Fitzgerald et al. (1999) infused Ang IV via the left renal artery and noted dose-dependent reductions in renal artery blood flow via transit-time flow probes placed around the artery. Once again these responses could be blocked by pretreatment with DuP753. Infusion of LVV-hemorphin-7 failed to alter blood flow. Presently it is difficult to resolve these conflicting results. Those reports noting Ang IV-mediated vasoconstriction determined that it was mediated by the AT_1 receptor type. Those reports noting Ang IV-induced vasodilation attributed it to the AT_4 receptor type. These Ang IV responses have been further separated into direct and indirect effects. Specifically, pretreatment with L-NAME prevented subsequent Ang IV-induced elevations in cerebral (Kramár et al., 1998) and renal cortical blood flow (Coleman et al., 1998). Nossaman et al. (1995) have shown that L-NAME and the cyclooxygenase inhibitor, meclofenamate, shift the Ang IV-induced vasoconstrictor dose-response curve in the pulmonary circulation. These researchers concluded that Ang IV could be promoting the release of vasodilators such as prostaglandins and nitric oxide. Consistent with these results, Yoshida et al. (1996) found the vasoconstriction induced by Ang IV to be initially facilitated by L-NAME, and by L-NAME and indomethacin (another cyclooxygenase inhibitor). In contrast, Li et al. (1997) have reported that indomethacin failed to impact the Ang IV dose-response curve in isolated human saphenous veins. Recently, Patel et al. (1998) and Hill-Kaputczak et al. (1999) determined that blockade of AT_1 and AT_2 receptor types failed to influence Ang II-induced NO release, or the release of NO synthase (NOS). Ang IV stimulated significantly greater NO release and greater endothelial-type constitutive NOS activity than the equivalent dose of Ang II. Divalinal-Ang IV blocked these Ang II and Ang II-mediated NO effects and NOS activation. The authors concluded that the Ang IV/ AT_4 system is primarily responsible for the Ang II facilitation of NO release and endothelium-dependent vasorelaxation. This endothelial cell NOS activation appears to be regulated by intracellular Ca^{2+} release and increased expression of calreticulin (Patel et al., 1999). Resolution of these issues awaits further investigation.

2. *Cardiac Hypertrophy.* Autoradiographic analyses utilizing ^{125}I -Ang IV reveals considerable binding in guinea pig and rabbit heart membranes that is distinct from AT_1 or AT_2 receptor types (Hanesworth et al., 1993). Angiotensin IV binding has also been shown using cultured vascular smooth muscle cells (Hall et al., 1993), coronary microvascular endothelial cells (Jarvis et al., 1992; Hall et al., 1995), and cultured rabbit cardiac fibroblasts (Wang et al., 1995b). Baker et al. (1990,

1992) have reported that Ang IV blocks increases in RNA and protein synthesis initiated by Ang II in chick cardiocytes. Rats, predisposed to left ventricular hypertrophy via abdominal aortic coarctation, revealed significant accumulations of collagen that is facilitated with the AT_1 receptor antagonist, divalinal-Ang IV, and significantly reduced when treated with the AT_4 receptor agonist *norleucine*³-Ang IV. These results point to a potentially important role for the Ang IV/ AT_4 system in collagen accumulation in hypertrophied hearts.

3. Renal Tubular Reabsorption. Autoradiograms utilizing ^{125}I -Ang IV demonstrate heavy distributions of AT_4 sites in the outer stripe of the outer medulla with diffuse labeling of the cortex (Coleman et al., 1998). This binding of Ang IV was not influenced by the specific AT_1 receptor antagonist losartan, or the AT_2 receptor antagonist PD123177. However, the AT_4 receptor antagonist, divalinal-Ang IV, or Ang IV, completely displaced this binding. Emulsion autoradiography determined that these AT_4 receptors are localized on cell bodies and apical membranes of convoluted and straight proximal tubules (Handa et al., 1998). Activation of these receptors by Ang IV produced a concentration-dependent decline in sodium transport as measured by rate of tissue oxygen consumption (Handa et al., 1998). Thus, these results provide strong evidence that Ang IV acts on tubular epithelium to inhibit sodium reabsorption. This natriuretic effect of Ang IV has been confirmed using *in vivo* infusion via the renal artery in anesthetized rats. Urine sodium concentration was found to significantly increase, whereas urine volume and glomerular filtration rate were not affected. This Ang IV-induced natriuresis was unaffected by pretreatment with DuP753 but was blocked with divalinal-Ang IV. These results support an important role for Ang IV in the control of sodium transport by the kidney (Ardailou and Chansel, 1996).

4. Electrophysiological Analysis. Albrecht and colleagues (1997a) have investigated the ability of micro-iontophoretic administration of Ang IV to influence neurons in the hippocampus and in the dorsal lateral geniculate nucleus (Albrecht et al., 1997b). Of 43 hippocampal neurons tested, Ang IV produced a greater than 40% increase in firing frequency in 16 of these cells (37%) with a decrease in three neurons. Of the 72 hippocampal neurons tested with Ang II, 21 evidenced a greater than 40% elevation in firing rate (29%), with a decrease in eight cells. With both Ang II and Ang IV the excitation effects were seen primarily in neurons that evidenced slower spontaneous discharge rates, whereas decreases in firing rate were usually seen in cells with higher spontaneous discharge rates. These changes in firing rates induced by Ang II could be blocked with the specific AT_1 receptor antagonist DuP753 (eight of ten neurons examined), however the specific AT_4 receptor antagonist divalinal-Ang IV was ineffective. On the other hand divalinal-Ang IV was effective at blocking

the Ang IV-induced increases in discharge rate in six of nine neurons tested, while DuP753 was ineffective. These results suggest that Ang II and Ang IV are acting at different receptors, i.e., AT_1 and AT_4 , respectively. Along these lines, the authors saw no evidence of cross-desensitization between Ang II and Ang IV. The authors suggest that the presumed colocalization of AT_1 and AT_4 receptor types on the same hippocampal neurons supports the notion that both Ang II and Ang IV are capable of influencing hippocampal neurons.

Similar findings were obtained with angiotensin-sensitive geniculate neurons (Albrecht et al., 1997b). These investigators further reported that Ang II application produced a potent inhibition of *N*-methyl-D-aspartate and kainate-induced facilitation of firing rates in some neurons, whereas increased discharge frequencies were observed in others. Ang IV was also found to block such glutamate receptor excitation in three neurons, whereas this excitation was facilitated in two neurons. Finally, these investigators found the iontophoretic application of Ang II onto these geniculate neurons suppressed light-evoked excitation (nine neurons) in some neurons, although other units also revealed facilitation (eight neurons). A similar evaluation of Ang IV indicated a facilitation of this light-evoked excitation in ten neurons and a suppression in seven neurons. These angiotensin effects could be blocked by the appropriate AT_1 or AT_4 receptor antagonists. Furthermore, the suppression effect of Ang II and Ang IV on light-evoked activity could itself be blocked by γ -aminobutyric acid receptor antagonists. Albrecht et al. (1976) suggested that Ang II and Ang IV appear to act as neuromodulators in the dorsal lateral geniculate nucleus. Determination of the precise role of each ligand will require further investigation.

5. Role of Ang IV in Learning and Memory. Intracerebroventricular injection of Ang IV leads to c-Fos expression in the hippocampus and piriform cortex, whereas similar injection of Ang II failed to induce c-Fos-like immunoreactivity in these structures but did activate c-Fos expression in circumventricular organs (Roberts et al., 1995) as well as paraventricular nucleus, SON, and the medial preoptic nucleus (Zhu and Herbert, 1996, 1997). Pretreatment with losartan prevented this Ang II-induced c-Fos-like immunoreactivity, whereas pretreatment with divalinal-Ang IV blocked the Ang IV-induced c-Fos expression (Roberts et al., 1995). There were no crossover effects demonstrated by these antagonists. Along these lines, Braszko and colleagues (1988, 1991) established that i.c.v. injected Ang II and Ang IV (1 nmol) were equivalent at facilitating exploratory behavior in rats tested in an open field, and furthermore, improved recall of passive avoidance conditioning and acquisition of active avoidance conditioning. Intracerebroventricular treatment with divalinal-Ang IV (10 nmol), disrupted recall of this response (Wright et al., 1995). Metabolically resistant analogs of Ang IV injected i.c.v. can be used to facilitate acquisition of successful

search patterns in a circular water maze task in scopolamine-treated rats (Pederson et al., 1998). Taken together, these results suggest an important role for the Ang IV/AT₄ system in learning and memory processes. Recently Mjølner et al. (1997) have isolated the globin fragment LVV-hemorphin-7, a decapeptide (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) that binds at the brain AT₁ receptor type, which could be the endogenous ligand that acts at this receptor.

Finally, Delorenzi and colleagues (1997) have reported Ang IV facilitation of long-term memory of an escape response in the crab *Chasmagnathus*. Angiotensin II was also shown to facilitate long-term memory in this species, although not as robustly; however, this Ang II effect could not be blocked by losartan or PD123177. These results suggest that the Ang IV/AT₄ system may have been involved in invertebrate memory processes long before the emergence of mammals.

E. Summary

Recently a new angiotensin receptor type, AT₄, has been discovered and characterized that preferentially binds Ang II(3-8), a fragment of angiotensin II, now referred to as Ang IV. This receptor site is prominent among brain structures concerned with cognitive processing, motor and sensory functions. Specifically, high densities of AT₄ sites have been localized in neocortex, piriform cortex, hippocampus, amygdala, and nucleus basalis of Meynert. Major motor structures with high levels of AT₄ receptors include basal ganglia, red nucleus, substantia nigra, ventral tegmentum, vestibular and reticular nuclei of the hindbrain, motor trigeminal nucleus, Betz cells of primary motor neocortex, cerebellum, and ventral horn of the spinal cord. Significant sensory structures include thalamus, the colliculi, gracile and cuneate nuclei, lateral geniculate, lateral olfactory tract, and primary sensory neocortex. Peripheral tissues that reveal heavy distributions of AT₄ sites are kidney, bladder, heart, spleen, prostate, adrenals, and colon.

The primary functions thus far associated with this Ang IV/AT₄ system include memory acquisition and recall, the regulation of blood flow, inhibition of renal tubular sodium reabsorption, and cardiac hypertrophy. There are preliminary indications that this system may also be involved in neurite outgrowth (Mjølner et al., 1996b), angiogenesis, and stimulation of endothelial cell expression of PAI-1 (Kerins et al., 1995). The identification of additional functions awaits further elucidation of this new receptor system. Cloning of the AT₄ receptor will certainly substantiate its potential role in pathophysiology of the renin-angiotensin system.

V. General Conclusions

Most of the known effects of Ang II are mediated through the AT₁ receptor, e.g., vasoconstriction, aldoste-

rone and vasopressin release, salt and water retention, and sympathetic activation without neglecting the important autocrine and paracrine effects of Ang II on cell proliferation and migration and on extracellular matrix formation. The function of the AT₂ receptor has become unraveled over the last few years owing to various sophisticated approaches including gene transfection and deletion. Accumulated published data suggests that the AT₂ receptor counterbalances the effect of the AT₁ receptor in vitro as well as in vivo. There is an inactivation of MAPK, antiproliferation, promotion of apoptosis, differentiation and regeneration, opening of voltage-rectifier K⁺ channels and closing of T-type Ca²⁺ channels. The re-expression of the AT₂ receptor in various diseases suggests a role of this receptor in pathophysiology.

The AT₄ receptor appears to be involved in memory acquisition and recall. Like the AT₂ receptor, it may also oppose the effect of the AT₁ receptor as it regulates renal blood flow, inhibits tubular sodium reabsorption and affects cardiac hypertrophy.

Cloning of the described angiotensin receptors and the ability to express these clones in mammalian cells will allow exhaustive structure/function studies. Further pharmacological and molecular studies will allow for a better and more complete understanding of the role of the renin-angiotensin system in pathology.

REFERENCES

- Abe J-I and Berk BC (1998) Reactive oxygen species as mediators of signal transduction in cardiovascular disease. *Trends Cardiovasc Med* 8:69-64.
- Abold RH and Harding JW (1988) Metabolism of angiotensin II and III by membrane-bound peptidases from rat brain. *J Pharmacol Exp Ther* 245:171-177.
- Adams R, Clovenstone TS, Wang X, and Murphy TJ (1999) Relationship between internalization and mRNA decay in down-regulation of recombinant type 1 angiotensin II receptor (AT₁) expression in smooth muscle cells. *Mol Pharmacol* 55: 1029-1036.
- Aguilera G, Capponi A, Baukai A, Fujita K, Haugler R, and Catt KJ (1979) Metabolism and biological actions of angiotensin II and des-Arg¹-angiotensin II in isolated adrenal glomerular cells. *Endocrinology* 104:1777-1785.
- Aguilera G, and Catt KJ (1978) Regulation of aldosterone secretion by the renin-angiotensin system during sodium restriction in rats. *Proc Natl Acad Sci USA* 75:4057-4061.
- Aguilera G, Haugler R, and Catt KJ (1978) Control of a aldosterone secretion during sodium restriction: adrenal receptor regulation and increased adrenal sensitivity to angiotensin II. *Proc Natl Acad Sci USA* 75:976-979.
- Aguilera G, Ryle CL, and Catt KJ (1982) Angiotensin II receptors and prolactin release in pituitary lactotrophs. *Endocrinology* 111:1045-1050.
- Aguilera G, Young WS, Kins A, and Butini A (1995) Direct regulation of hypothalamic corticotropin-releasing-hormone neurons by angiotensin II. *Neuroendocrinology* 61:437-444.
- Almehd A, Li XP, Shams M, Gregory J, Rossman T, Barnes NM and Newton JR (1996) Localization of the angiotensin II and its receptor subtype expression in human endometrium and identification of a novel high-affinity angiotensin II binding site. *J Clin Invest* 96:848-857.
- Aiyar N, Baker E, Follen M, Nishikawa P, Burgoyne DJ, Kumar C and Nambi P (1994) Characterization of a functional angiotensin II receptor in Xenopus laevis heart. *Pharmacology* 48:242-249.
- Aiyar N, Baker E, Wu H, Nambi P, Edwards RL, Tietz JL, Ellis C and Burgoyne DJ (1994) Human AT₂ receptor is a single copy gene: Characterization in a stable cell line. *Mol Cell Biochem* 131:75-86.
- Albrecht D, Brower MK and Kruger H (1997a) Excitatory action of angiotensin II and IV on hippocampal neuronal activity in urethane anesthetized rats. *Regul Pept* 70:105-109.
- Albrecht D, Brower MK, Kruger H and Bader M (1997b) Effects of angiotensin II and IV on granule activity in zosterangiotensin and transgenic rats. *Eur J Pharmacol* 330:55-63.
- Alonso ML, Leger CL, Rasooljanahy J, Wandschew DZ, Clausner H, Enjalbert A and Kardon G (1994) Selective effect of a des-Arg¹-angiotensin II on the arachidonic acid membrane-phospholipid content on in vitro phospholipase C and adenylate cyclase-mediated pituitary response to angiotensin II. *Neuroendocrinology* 60: 400-406.
- All MS, Sayanski PP, Dirksen LB, Hayer DJ, Marrero MB and Bernstein KB (1997a) Dependence on the motif Y127 for the physical association of Val¹ kinase with the intracellular carboxyl tail of the angiotensin II AT₁ receptor. *J Biol Chem* 272: 33592-33598.

- Al HS, Schiefer B, Dolanowski J, Bernstein RJ, Ling HN and Marrero MB (1997b) Angiotensin II stimulates tyrosine phosphorylation and activation of insulin receptor substrate 1 and protein-tyrosine phosphatase L2 in vascular smooth muscle cells. *J Biol Chem* 272:12372-12379.
- Allen AM, MacGregor DP, McInnes P and Mendelsohn FAO (1999a) Angiotensin II receptors in the human brain. *Neurosci Lett* 791-7.
- Allen AM, Moeller J, Jenkins TA, Zhou J, Alred GP, Chai S-Y and Mendelsohn FAO (1999b) Angiotensin receptors in the nervous system. *Brain Res Bull* 47:17-26.
- Allen AM, Oldfield BJ, Gille ME, Paxinos G, McKinley MG and Mendelsohn FAO (1999b) Localization of angiotensin receptors in the nervous system. *Handbook of Chemical Neuroanatomy*, in press.
- Amanat C, Tancos M, Barreau C, Ribard F, Falgaue N, McFadden EP, Baudoux X, Lalonde JM, Amouyel P and Bertrand ME (1997) The angiotensin II type 1 receptor gene polymorphism is associated with coronary artery vasorestriction. *Am J Cardiol* 80:295-299.
- Amoroso C and Catt KJ (1992) Angiotensin II receptor-mediated calcium influx in bovine adrenal glomerulosa cells. *Endocrinology* 131:408-414.
- Amoroso C, Clark AJL and Catt KJ (1991) The mas oncogene encodes angiotensin-induced (Ca^{2+}) responses in cells with pre-existing angiotensin II receptors. *Biochim Biophys Acta* 1133:107-111.
- Amulali P, Felix D, Imboden H, Khosla M and Ferrario CM (1992a) Effects of angiotensin II and its selective antagonist on inferior olivary neurons. *Regul Pept* 41:19-26.
- Amulali P, Felix D, Imboden H, Khosla M and Ferrario CM (1992b) Effects of angiotensin analogues and angiotensin receptor antagonists on paraventricular neurons. *Regul Pept* 38:111-120.
- Anderson KM, Maruhashi T, Dostal DE and Peach MJ (1983) Morphological and biochemical analysis of angiotensin II phosphorylation in cultured rat aortic smooth muscle cells. *Am J Physiol* 246:C179-C188.
- Arvidsson R and Chamei D (1995) Angiotensin IV, a new component of the renin-angiotensin system, which acts on kidney cells. *Eur J Acad Natl Med* 180:474-495.
- Armstrong DL, Garcia EA, Ma T, Quisenberry B and Wyner MJ (1998) Angiotensin II blockade of long-term potentiation at the perforant path-granule cell synapse in vitro. *Peptides* 17:689-695.
- Audouze V, Ramiandrianja R, Bertrand P, Pivian M, Kordon C and Enjalbal A (1991) Involvement of protein kinase-C in the effect of angiotensin-II on adenosine 3',5'-monophosphate production in lutealroph cells. *Endocrinology* 129:2231-2239.
- Auzanias A, Sakarellos G, Lintner K, Fernandez S, Khosla MK, Smey R and Bumpus PM (1988) Studies on angiotensin II and analogs: impact of substitution in position 6 on activity and selectivity. *Proc Natl Acad Sci USA* 85:1881-1885.
- Baker KM and Azeite JF (1990) Angiotensin II stimulation of protein synthesis and cell growth in chick heart cells. *Am J Physiol* 259:H1010-H1018.
- Baker KM, Booz GW and Dostal DE (1992) Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. *Am J Physiol* 262:H227-H231.
- Baldwin JM, Scherler GF and Unger WJ (1997) An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. *J Mol Biol* 270:141-164.
- Barak LS, Thierl M, Freedman NJ, Kwastman M, Teitelbaum RJ and Caron MG (1998) A highly conserved residue in G-protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. *J Biol Chem* 273:2790-2795.
- Barber MM, Sampson DB and Wilder RD (1990) AT₁ receptor stimulation enhances antihypertensive effects of A₂ receptor antagonist in hypertensive rats. *Hypertension* 9:411-417.
- Beukel AJ, Hunyady L, Catt KJ and Balle T (1994) Evidence for participation of calcineurin in potentiation of angiotensin-stimulated Ca^{2+} formation by the calcium-mobilizing hormone, angiotensin II. *J Biol Chem* 269:6445-6448.
- Bleu-Villanar D, Lucas-Mongelli JM, Thyssen SM, Diaz-Torges GS and Liberman C (1994) Effects of LHRH and ANG II on prolactin stimulation are mediated by hypophyseal AT₁ receptor subtypes. *J Physiol* 206:374-379.
- Bledsoe K, Ebbas N, Sauton M, Masson M, Suaini G, Strohberg AD and Nahmias C (1997) Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase activity and functional activation of SHP-1 tyrosine phosphatase. *Biochem J* 325:449-454.
- Boggett M, Jongleis D, Vilgrain J and Saez JM (1997) Angiotensin II (A₁) steroidogenic reductase in Y-1 cells in the presence of A-7 receptors negatively coupled to adenylyl cyclase. *Endocrinology* 140:3501-3516.
- Borek RH, Olpe HR, Jones RB and Edozhanov AG (1994) Microregulation of vasopressin into the locus coeruleus of conscious rats. *Am J Physiol* 267:H675-H683.
- Borgeson DJ, Ellis C, Kumar C, Nishikant P, Kortein H, Eshaghy N, Carlin E, Stedel JM and Aiyar N (1982) Cloning and characterization of a human angiotensin II type 1 receptor. *Biochem Biophys Res Commun* 103:889-895.
- Berk BC and Cornwell PE (1997) Angiotensin II signal transduction in vascular smooth muscle: Role of tyrosine kinases. *Curr Res* 8:607-615.
- Berk BC, Velevich V, Gordon HM and Teude T (1989) Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension* 13:305-314.
- Bernier SG, Servant G, Goudreau M, Fournier A and Guilleminet G (1995) Characterization of binding site for angiotensin IV on bovine aortic endothelial cells. *Eur J Pharmacol* 281:157-160.
- Bernstein KB and Marrero MB (1996) The importance of tyrosine phosphorylation in angiotensin II signaling. *Trends Cardiovasc Med* 6:179-187.
- Berridge MJ, Booter MD and Lipp P (1998) Calcium - a life and death signal. *Nature (Lond)* 395:645-648.
- Bhat GJ, Abraham ST and Baker KM (1996) Angiotensin II interferes with interleukin-6-induced NF- κ B signal transduction involving mitogen-activated protein kinase kinase-1. *J Biol Chem* 271:24247-24252.
- Bhat GJ and Baker KM (1997) Angiotensin II stimulates rapid serine phosphorylation of transcription factor NF- κ B. *Mol Cell Biochem* 170:173-178.
- Bhat GJ, Thakurkumar TJ, Thomas WG, Conrad KM and Baker KM (1994) Angiotensin II stimulates cis-acting factor-like DNA binding activity. Evidence that the AT₁ receptor activates transcription factor-Stat1 and/or a related protein. *J Biol Chem* 269:31443-31449.
- Bhat GJ, Thakurkumar TJ, Thomas WG, Conrad KM and Baker KM (1995) Activation of the STAT pathway by angiotensin II in TSC2/STAT₁ cells. Cross-talk between angiotensin II and interleukin-6 nuclear signaling. *J Biol Chem* 270:19669-19678.
- Birkmeier CA and Buckley JP (1981) Evidence for a central mechanism of angiotensin-induced hypertension. *Proc Soc Exp Biol Med* 168:634-637.
- Björnsen C, Mannot C, Davies E, Teusch B, Barnstein KR, Corvol P and Clausen B (1993) Mutation of Asp74 of the rat angiotensin receptor gene causes changes in autonomic regulation and abolishes G-protein coupling. *Proc Natl Acad Sci USA* 90:5128-5137.
- Birbaeas MA, Coppola AM and Vallarini MB (1984) Solubilized adrenal angiotensin II receptors on the site of action of sodium and calcium ions, and on the role of disulfide bridges. *Mol Cell Endocrinol* 37:181-189.
- Bleuel A, de Gasparo M, Whitebread S, Purrier J and Monard D (1995) Regulation of protein kinase C expression in cultured Schwann cells is mediated by angiotensin II receptors. *J Neurosci* 15:7670-7671.
- Blume A, Herdendorf T and Unger T (1998) Angiotensin peptides and inducible transcription factors. *J Mol Med* 77:559-557.
- Boucard A, Davies E, Jamarate X, Fery I, Charu A, Clausen E, Tivet L, Cambien F, Corvol P and Souvrier F (1994) Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. *Hypertension* 24:652-659.
- Booz GW, Conrad KM, Eess AJ, Singer HA and Baker KM (1992) Angiotensin-II-binding sites on hepatocyte nuclei. *Endocrinology* 130:3041-3049.
- Boeckelmann D, Oser NK, Moser U and Aasi A (1995) Tenofovir and 6-hydroxy-chloroquine inhibit angiotensin II-induced vascular smooth muscle cell proliferation by a nonantagonist mechanism. *Arch Biochem Biophys* 318:941-946.
- Boess R, Gerold M, Fischl W, Holak M and Reicher E (1990) An angiotensin with prolonged action: blood pressure-lowering properties. *J Cardiovasc Pharmacol* 18 (Suppl 3):950-955.
- Bottari SP, King IN, Reichlin S, Delbostre L, Lydon N and de Gasparo M (1992) The angiotensin AT₁ receptor: stimulates protein tyrosine phosphatase activity and mediates inhibition of particulate guanine cyclase. *Biochem Biophys Res Commun* 182:205-211.
- Bottari SP, Taylor V, King IN, Bogdan V, Whitebread S and de Gasparo M (1991) Angiotensin II AT₁ receptors do not interact with guanine nucleotide binding proteins. *Eur J Pharmacol* 207:167-163.
- Boulay G, Chretien L, Richard DF and Guilleminet G (1994) Short-term desensitization of angiotensin II receptor of bovine aortic glomerular cells corresponds to a shift from a high to a low affinity state. *Endocrinology* 135:2190-2196.
- Bouvard L, Blackmore PF and Dutton JH (1985) Characterization of the angiotensin II receptor in primary cultures of rat hepatocytes. Evidence that a single population of receptors is responsible for receptor-mediated effects. *Endocrinology* 116:1401-1409.
- Brazzo JJ, Kupresewicz A, Wlascak B and Wisniewski K (1988) Angiotensin II (3-8)-hexapeptide affects motor activity, performance of passive avoidance and a conditioned avoidance response in rats. *Neuroscience* 27:777-783.
- Brazzo JJ, Wisniewski K, Kononickiewicz W, Jenczak A and Wisniewski K (1991) The 3-fragment of angiotensin II is probably responsible for its psychomotor properties. *Neurosci Res* 8:49-54.
- Braun-Munzel E, Fasilco JC, Leloir LF and Munoz JM (1990) The substance causing renal hypertension. *J Physiol* 498:283-288.
- Bredtner V, Jones PV, Lavens NM, de Gasparo M and Bottari SP (1998) Angiotensin and angiotensin peptides in angiotensin analogs at the AT₁ receptor in PC12W cells. *Regul Pept* 84:207-213.
- Brilla CG, Remes GP, Maizels B and Weber KT (1993) Renin-angiotensin system and myocardial collagen in hypertension: Regulation of the myocardial collagen matrix. *Regul Pept* 44:207-213.
- Brilla CG, Rupp H, Funck R and Maizels B (1995a) The renin-angiotensin-aldosterone-mitogenic pathway: collagen matrix remodeling in congestive heart failure. *Eur J Heart* 17:107-109.
- Brilla CG, Zhou G, Rupp H, Maizels B and Weber KT (1995b) Role of angiotensin II and prostaglandin E₂ in regulating cardiac fibroblast collagen turnover. *Am J Cardiol* 76:80-100.
- Brink M, Erne P, de Gasparo M, Regg H, Schmid A, Stolz P and Bollock G (1996) Localization of the angiotensin II receptor subtypes in the human adrenal. *Am J Cardiol* 78:1329-1339.
- Brown NJ, Agbireh M and Vaughan DE (1990) Comparative effect of angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor antagonism on lung. *Eur Respir J Suppl* 86:285-290.
- Buisson B, Bottari SP, de Gasparo M, Galis-Payet N and Payet MD (1992) The angiotensin AT₁ receptor modulates T-type calcium current in non-differentiated neuroblastoma SK-N-BE cells. *FEBS Lett* 309:161-164.
- Buisson B, Lafont LA, Bottari SP, de Gasparo M, Galis-Payet N and Payet MD (1995) A G-protein is involved in the angiotensin AT₁ receptor inhibition of the T-type calcium current in non-differentiated NG108-15 cells. *J Biol Chem* 270:1670-1674.
- Bullock GR, Steynart I, Carey R, Siragy H, Bilbe G, Prset M and de Gasparo M (1999) Distribution of angiotensin II type 1 and type 2 receptors in the human lung. *Eur Respir J Suppl* 86:291-292.
- Bumpus PM (1997) Mechanisms and sites of action of newer angiotensin agonists and antagonists in terms of safety and receptor. *Fed Proc* 36:2128-2132.
- Burns DJ, Chiu KJ, de Gasparo M, Goodfriend T, Yussif A, Peach MJ, Taylor DO and Zimmerman PMW (1991) Nonantagonist for angiotensin receptors. *Hypertension* 17:720-721.
- Burns DJ, Chiu KJ, de Gasparo M and Page JE (1997) Synthesis and pharmacology of the octapeptide angiotensin. *Science (Wash DC)* 276:886-887.
- Burns DJ, Inagami T and Harris RO (1993) Cloning of a rabbit kidney cortex AT₁ angiotensin II receptor that is present in proximal tubule epithelium. *Am J Physiol* 265:F546-F554.

- de Gaspero M, Whitebread D, Kalsner MK, de Herrogh J, Crevolter P and Thomas van der Griend A (1984) Down regulation of the angiotensin II receptor subtype AT₂ in human myocardium during pregnancy. *Regul Pept* 63:39–45.
- de Gaspero M, Whitebread D, Kalsner MK, Whitebread PJ, Ramonowicz P and Kalsner B (1990) Biochemical characterization of two angiotensin II receptor subtypes in rat. *J Cardiovasc Pharmacol* 16 (Suppl 4):331–36.
- De Lenn A, Ong H, Gutkowska J, Sciallari PW and McNeill N (1984) Evidence for agonist-induced internalization of angiotensin II receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. *Mol Pharmacol* 26:584–593.
- Diesel C (1986) Paracrine interactions in the anterior pituitary. *Clin Endocrinol Metab* 25:1–32.
- Della Brana R, Riet S, Himmelseisen C and Kurts A (1995) Expression of cardiac angiotensin II AT₁ receptor genes in rat hearts is regulated by steroids but not by angiotensin II. *J Hypertens* 13:763–769.
- Dellon AL, Leontiev T, Roman A, Nohmed V and Maldonado H (1997) Angiotensin II (8–21) induces long-term memory improvement in the crab *Chasmagnathus*. *Neurosci Lett* 226:133–135.
- De Oliveira AM, Viswanathan M, Hasekrajek FMJ, Correa FMA and Savenkov JM (1994) Specific non-peptide angiotensin II [¹²⁵I]-[DAP1211] binding sites in rat spleen membranes. *Biochem Biophys Res Commun* 200:1048–1058.
- Delvalle ES, Humeau A, Suley RT and Kastrup PA (1988) Measurements of immunoreactive angiotensin peptides in rat tissues: Some pitfalls in angiotensin II analysis. *Anal Biochem* 174:80–87.
- Derynck M and Meyer P (1978) Angiotensin receptors. *Biochem Pharmacol* 27:1–5.
- Diaz-Torga G, Gonzalez Iglesias A, Alvarez-Zea R, Libertun G and Benj-Villalobos D (1998) Angiotensin II-induced Ca²⁺ mobilization and protein release in normal and hyperplastic pituitary cells. *Am J Physiol* 274:E334–E340.
- Diaz-Torga GS, Benj-Villalobos D and Libertun G (1994) Outflow of angiotensin II-induced prolactin release: in vivo and in vitro in female and male rats. *Neuroendocrinology* 58:57–62.
- Dinarelle S, Rippmann Y, Weiland U, Haendeler J and Zeiler AM (1997) Angiotensin II induces apoptosis of human endothelial cells: Protective effect of calcitonin. *Circ Res* 81:970–978.
- Dohmann HG, Chazotte MC, Badiou A, Friele T and Lefkowitz RG (1990) Role of the beta 2-adrenergic receptor in the binding of the ligand binding function of the beta 2-adrenergic receptor. *Proc Natl Acad Sci USA* 87:2335–2342.
- Douglas D, Findlay JB and Blundell TL (1994) The evolution and structure of aminergic G protein-coupled receptors. *Receptors Channels* 3:61–76.
- Dostal DE and Baker KM (1993a) Angiotensin II stimulation of left ventricular hypertrophy in adult rat heart. *Am J Hypertens* 6:278–280.
- Dostal DE, Bone GW and Baker KM (1993b) Angiotensin II signaling pathways in cardiac fibroblasts: Contradictory versus novel mechanisms in mediating cardiac growth and function. *Mol Cell Biochem* 117:21–25.
- Dostal DE, Robinson KM, Czerwik ML, Cooper GP and Baker KM (1995b) Intracellular detection of angiotensin II in the ligand and renin-angiotensin system in neonatal rat heart. *Am J Physiol* 268:C838–C850.
- Dougherty J and Cui J (1976) Regulation of angiotensin II receptors in the rat adrenal cortex by dietary electrolytes. *J Clin Invest* 80:934–941.
- Douglas JG (1987) Angiotensin receptor subtypes of the kidney cortex. *Am J Physiol* 253:F37–F47.
- Dudley DT, Panek RJ and Major TO (1990) Subclasses of angiotensin II binding sites and their functional significance. *Mol Pharmacol* 58:370–377.
- Dudley DT, Hubbell SE and Summerfield RM (1991) Characterization of angiotensin II (AT₂) binding sites in RHT cells. *Mol Pharmacol* 40:390–397.
- Dudley DT and Summerfield RM (1988) Repeated expression of angiotensin II (AT₂) binding sites in RHT cells. *Regul Pept* 24:199–206.
- Dulin NO, Rennerberg P, Stein JD and Douglas JG (1994) Rabbit renal epithelial angiotensin II receptors. *Am J Physiol* 267:F772–F778.
- Dulin N, Madhavi NT, Chang CL, Bert-Matthews L, Dickerson D and Douglas JG (1995) Angiotensin II receptors and signaling in opossum kidney cells. *Am J Physiol* 269:F454–F459.
- Dutur P, Bazzani MJ, Serut MC and Lamour Y (1995) The septal cholinergic pathway: Structure and function of a central cholinergic system. *Physiol Res* 76:489–497.
- Dzau VJ, Baxter JA, Cazin M, de Boff A, Ganim D, Gross H, Husein A, Inagami T, Menard J, Peole S, Robertson JJ, Tang J and Yamamoto K (1987) Report of the Joint Nomenclature and Nomenclature Committee of the International Society of Hypertension, American Heart Association and the World Health Organization. *Hypertension* 5:607–611.
- Dzau VJ and Chikara JM (1987) Autoregulation and paracrine mechanisms of vascular myocytes in systemic hypertension. *Am J Cardiol* 59:991–1001.
- Eguchi S, Matsumoto T, Motley ED, Utsumiya H and Inagami T (1996) Identification of an essential signaling cascade for mitogen-activated protein kinase activation by angiotensin II in cultured rat vascular smooth muscle cells. *J Biol Chem* 271:14169–14175.
- Eguchi S, Numaguchi K, Iwase H, Matsumoto T, Yamakawa T, Utsumiya H, Motley ED, Kawasumi H, Inagami T and Inagami T (1995) The angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J Biol Chem* 270:5950–5956.
- Elliot DJ and Peart WS (1956) Amino acid sequence in a hypertensive. *Nature (Lond)* 177:527–528.
- Engeli S, Gnanapavan R, Kraus R, Junkel N, Distler A and Sharma AM (1999) Co-expression of renin-angiotensin system genes in human adipose tissue. *J Hypertens* 17:655–660.
- Erbenberg P, Zhou J, Dorman T and Douglas JG (1995) Angiotensin II receptor subtypes in cultured rat angiotensin II receptor transfection mediated by the bacteriophage. *Pharmacol Biochem Behav* 40:261–268.
- Erbenberg P, Zhou J, Marino EM, Goddard JM, Cappelletti M and Bernstein KJ (1996) Mice lacking angiotensin-converting enzyme have blood pressure, renal pathology, and vascular remodeling. *Lett Invest* 74:555–562.
- Erbenberg P, Marino EM, Howard BS, Andrews A, Corvo P, Cappelletti M and Bernstein KJ (1997) The critical role of tissue angiotensin-converting enzyme as revealed by gene targeting in mice. *J Clin Invest* 100:107–118.
- Felix D and Alvert K (1974) The effect of angiotensin II on neurons of the cat subfornical organ. *Brain Res* 76:350–353.
- Felix D and Harding JW (1988) Manipulation of aminopeptidase activities: differential effects of immunoregulatorily supplied angiotensin in rat brain. *J Hypertens* 4:398–401.
- Felix D, Harding JW and Imboden H (1988) The hypothalamic-angiotensin system: functional and clinical considerations. *Clin Exp Hypertens (A)* 10 (Suppl 1):45–62.
- Felix D and Schlegel W (1978) Angiotensin receptor neurons in the subfornical organ: relationship to central considerations. *Brain Res* 167:119–126.
- Feng Y-H, Mura B, Hsuan A and Karmali SS (1998) Mechanism of constitutive activation of the AT₁ receptor: influence of the size of the agonist versus binding. *Endocrinology* 142:1779–1785.
- Feng Y-H, Noda K, Saei Y, Liu XP, Hsuan A and Karmali SS (1996) The cloning of Arg2 of angiotensin II with Asp281 of AT₁ receptor is essential for full agonism. *J Biol Chem* 270:12646–12650.
- Ferrario AV and Balza JS (1987) Actions of angiotensin in the subfornical organ and area postrema: Implications for long term control of autonomic output. *Clin Exp Pharmacol* 14:506–101.
- Ferrario CM, Brumini LB, Dix DJ, Jaiswal N, Khosla MC, Miletic A and Tallant EA (1991) Angiotensin (1–7): A new hormone of the angiotensin system. *Hypertension* 18 (Suppl 3):123–133.
- Ferrario CM and Dix SN (1998) Angiotensin (1–7): A bioactive fragment of the renin-angiotensin system. *Regul Pept* 78:13–18.
- Feuillan PF, Millan MA and Aquilar G (1983) Angiotensin II binding sites in the rat: site characterization of receptor subtypes and interaction with guanylyl nucleotides. *Regul Pept* 44:161–169.
- Flecken FL, Vanderheyden PM, De Backer JP and Vauquelin G (1999) Immunoreactive angiotensin AT₁ receptor antagonists: The role of tight antagonist binding. *Eur J Pharmacol* 370:189–205.
- Findlay JB, Donnelly D, Bhargal N, Hurrell C and Atwood TK (1993) Structure of G-protein-linked receptor. *Biochem Soc Trans* 21:869–873.
- Fischer S, St (1998) The renin-angiotensin system: A novel expression in human endothelial cells following stimulation of AT₁ receptors. *J Vasc Res* 35 (Suppl 1):26.
- Fisher PM, Rahman Y, Gelstein JD and Finkelman S (1971) Angiotensin and renin in the dog brain. *J Exp Med* 133:553–561.
- Fitzgerald SM, Evans RG, Bergstrom G and Anderson WP (1999) Renal hemodynamic response to intravenous infusion of ligands for the putative angiotensin IV receptor in anesthetized rats. *J Cardiovasc Pharmacol* 34:200–211.
- Forio T, Fan MG, Newman B, Hershberger RP, Civelli O and Strick P (1992) Dopamine-regulated inhibition of DNA synthesis in pituitary tumor cells is associated with phosphatidylinositol 3-kinase and protein kinase C. *J Neuroendocrinol* 4:172.
- Fortuno MA, Ravassa S, Eryoz G and Dix J (1998) Overexpression of Ras protein and enhanced apoptosis in the left ventricle of spontaneously hypertensive rats – effects of AT₁ blockade with losartan. *Hypertension* 32:280–283.
- Freix JED (1995) Historical development of antihypertensive treatment. In: *Hypertension: Pathophysiology, Diagnosis, and Management* (Larragh JH and Brenner BM eds) pp 274–275. Raven Press Ltd, New York.
- Freudenreich SM, Schmitt T, Leuchs I and Glatzer CH (1999) Furosemide stimulates human erythropoietin production via activation of the renin-angiotensin system. *Br J Clin Pharmacol* 48:631–634.
- Friberg P, Sundelin B, Bolander S-O, Bock A, Nilsson H, Wikman A, Gustafsson B, Petersen J and Adams M (1995) Renin-angiotensin system in neonatal rats: Induction of a renal angiotensin II response to ACE inhibition or angiotensin II antagonist. *Kidney Int* 48:456–462.
- Fu ML, Schulze W, Walukut G, Ellis R, Eftekhari P, Kjalmarsson A and Edeboke J (1998) Immunohistochemical localization of angiotensin II receptors (AT₁) in the heart with anti-peptide antibodies showing a positive chromogenic effect. *Recept Channels* 6:99–111.
- Furukawa Y, Kishimoto S and Nishikawa T (1982) Lipoxygenase: imidazole derivatives. US Patent 4,340,558 issued to Takeda Chemical Industries, Ltd, Osaka, Japan.
- Furuta H, Guo DF and Inagami T (1992) Molecular cloning and sequencing of the gene encoding human angiotensin II type 1 receptor. *Biochem Biophys Res Commun* 188:1–10.
- Gaborik Z, Mihailik B, Jayes B, Jagadeesh G, Cett KJ and Huynh LD (1996) Requirement of membrane-proximal arylamine acid in the carboxyl-terminal tail for angiotensin II receptor activation. *FEBS Lett* 428:147–151.
- Gallinat S, Basche S, Schiller S, Kramel S, Kramel M and Unger T (1999) AT₁ receptor stimulation induces generation of ceramide in PC12W cells. *FEBS Lett* 443:75–78.
- Gallinat S, Calkins D, MacLennan S, Hargrove T, Stoll M and Unger T (1997) The angiotensin AT₁ receptor down-regulates neuronal nitric oxide in PC12W cells. *Neurosci Lett* 227:29–32.
- Gallinat S, Yu M, Decker A, Unger T and Hargrove T (1998) Sarcosine reverse transcription evokes latent up-regulation of angiotensin AT₁ and AT₂ receptor mRNA in adult rat dorsal root ganglia and sciatic neurons. *Brain Res* 817:111–123.
- Garcia-Cardena P (1994) The brain renin-angiotensin system and its role in the regulation of secretion of anterior pituitary gland. *Front Neuroendocrinol* 14:238–249.
- Garcia-Cardena P and Seldin DF (1973) The intrinsic brain renin-angiotensin system: Its possible role in central mechanisms of blood pressure. *Clin Med* 48:265–268.
- Ganten D and Speck G (1978) The brain renin-angiotensin system: A model for the synthesis of angiotensin in the brain. *Biochem Pharmacol* 27:3273–3283.
- Gardiner SM, Kemp PA, March JE and Bennett T (1993) Regional haemodynamic effects of angiotensin II (8–21) in conscious rats. *Br J Pharmacol* 110:156–162.
- Goss JM, Shanmugasundaram S, Sibany M and Corvel P (1994) Tissue-specific expression of

- type 1 angiotensin II receptor subtypes. An *in situ* hybridization study. *Hypertension* 24:531-537.
- Gohblert DR, Gockenheuser SL and Schöber D (1991a) Angiotensin II receptor subtypes in rat brain: Differential binding to A₁- and A₂-type and subtypes binding to A₁. *Brain Res* 54:161-165.
- Gohblert DR, Gockenheuser SL and Schöber D (1991b) Autoradiographic localization of subtypes of angiotensin II antagonist binding in the rat brain. *Neuroscience* 44:501-514.
- Gohblert DR, Speth RO and Wenzel KJ (1989) Quantitative autoradiography of angiotensin II receptors in SHR brain. *Physiol* 73:1051-1057.
- Gokhale AA, Rauch MJ and Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res* 62:749-755.
- Gondouin L, Lafamme I, Rivard N, Asselin C, Payet MD and Gellé-Payet N (1989) Signals from the AT₁ (angiotensin type 1) receptor of angiotensin II inhibit p21^{ras} and activate MAPK (mitogen-activated protein kinase) to induce morphological, neuronal differentiation in NG108-15 cells. *Mol Endocrinol* 18:1615-1626.
- Guthier U and Kobilka BK (1995) Protein-coupled receptors. II. Mechanism of agonist activation. *J Biol Chem* 270:17973-17979.
- Olsson RE, Thorpe HN, Corvettie ME, Frank JD, Short TW, Bunting PS and Siegel PKG (1991) Angiotensin II receptor subtypes in renal cortex of rats and sheep. *Nucleic Acids Res* 19:1512-1518.
- Gillis JC and Muehlman A (1987) Interactions, a review of the pharmacodynamic and pharmacokinetic properties and therapeutic use in the management of hypertension. *Drugs* 34:885-892.
- Glossmann H, Bunkal A and Catt KJ (1974a) Angiotensin II receptors in bovine adrenal cortex. Modification of angiotensin II binding by guanylin nucleotides. *J Biol Chem* 249:684-686.
- Glossmann H, Bunkal A and Catt KJ (1974b) Cation dependence of high-affinity angiotensin II binding to adrenal cortex receptors. *Science (Wash DC)* 182:381-383.
- Glossmann H, Bunkal A and Catt KJ (1974c) Properties of angiotensin II receptors in the bovine and rat adrenal cortex. *J Biol Chem* 249:825-831.
- Gos KL and Wagnat AJ (1990) Laser Doppler flowmetry in the study of pharmacology, clinical efficacy and tolerability in the management of hypertension. *Drugs* 51: 800-845.
- Gokhale P, Pace C and Unger T (1993) AT₁ receptor stimulation increases cyclic GMP in SHRSP by a nitric-dependent mechanism. *Hypertension* 21:549-555.
- Goldblatt H, Lynch J, Hanzel RF and Summerville WW (1934) Studies on experimental hypertension. I. The production of persistent elevation of systolic blood pressure by means of renal ischemia. *J Exp Med* 59:347-349.
- Goldfarb DA, Dix DJ, Tubbs RE, Ferruccio CM and Novack AC (1994) Angiotensin II receptor subtypes in the human renal cortex and renal cell carcinoma. *J Urol* 151:208-213.
- Goldman J and Cote L (1991) Aging of the brain: Dimensions of the Alzheimer's type, in *Principles of Neural Science*, (Kandel ER, Schwartz JH and Jessell TM eds) pp 974-988, Elsevier Science Publishing, New York.
- Goodfield NF, Newby DJ, Lallan CA and Flapan AD (1999) Effects of acute angiotensin II type 1 receptor antagonism and angiotensin converting enzyme inhibition of plasma fibrinolytic parameters in patients with heart failure. *Circulation* 99:2585-2595.
- Goodfield NF, Elliott ME and Catt KJ (1995) Angiotensin receptors and their antagonists. *New Engl J Med* 344:1548-1554.
- Goodfield NF and Lin SY (1997) Receptors for angiotensin I and II. *Circ Res* 79:163-174.
- Goto M, Mukoyama M, Suga S, Matsumoto T, Nakagawa M, Ishihara R, Kishihara M, Sugawara I, Tanaka I and Nakao K (1997) Growth-dependent induction of angiotensin type 2 receptor in rat mesangial cells. *Hypertension* 30:558-562.
- Govantes C and Marín J (1998) Effect of angiotensin converting enzyme inhibitors on quality of life in hypertensive patients. *Pharmacokinetic basis*. *Fundam Clin Pharmacol* 10:400-405.
- Grady RF and Kallay JB (1993) Expression of AT₁ receptors in rat fetal subdermal cells. *Regul Pept* 44:173-180.
- Grady RF, Smith LA, Griffin CA, Schenbaum M and Kallay JB (1991) Expression of AT₁ receptors in the developing rat fetus. *J Clin Invest* 88:821-833.
- Gröndberg RF, Uhlöf-Pukki M, Lasseus B and Alexander RW (1997) Angiotensin II signaling in vascular smooth muscle: New concepts. *Hypertension* 29:366-373.
- Griffin SA, Brown WC, MacLachlan P, McGrath JC, Wilson WG, Kousgaard N, Mulvany MW and Lever AP (1991) Angiotensin II causes vascular hypertrophy in part by a non-proteinase mechanism. *Hypertension* 17:698-705.
- Grubisova T, Maigret B, Nouse S, Languier R, Lombard C, Bannaud JC and Marie J (1990) Amino acids of the third transmembrane domain of the AT₁ angiotensin II receptor are involved in the differential recognition of peptide and nonpeptide ligands. *Biochem Biophys Res Commun* 200:158-160.
- Grüne EJ, Simon M and Fuchs E (1999) Autoradiographic characterization of angiotensin receptor subtypes in fetal and adult human kidney. *Am J Physiol* 269:F399-F401.
- Gress F (1988) The regulation of aldosterone secretion by the renin-angiotensin system under various conditions. *Acta Endocrinol (Copenh)* (Suppl) 134:1-41.
- Gross DJ, Purita H, Mironakshi M and Inagami T (1994) The genomic organization of human angiotensin II type 1 receptor. *Biochem Biophys Res Commun* 200:313-319.
- Guo DF and Inagami T (1994b) Epidermal growth factor-enhanced human angiotensin II type 1 receptor. *Hypertension* 23:1032-1035.
- Guo DF, Guo S and Inagami T (1996) Steroid hormone upregulate rat angiotensin II type 1A receptor gene: Role of glucocorticoid responsive elements in rat angiotensin II type 1A promoter. *J Steroid Biochem Mol Biol* 53:69-78.
- Gyuhko R, Kikuchi B, Kusanagi F, Owens FT and Phillips MJ (1992) Angiotensin II receptor subtypes play opposite roles in regulating phosphatidylinositol hydrolysis in rat skin cells. *Biochem Biophys Res Commun* 188:295-297.
- Haber RL (1994) Role of angiotensin receptor subtypes in the response of rabbit brain arteries to angiotensin. *Stroke* 25:1476-1480.
- Haber RL, Anasae F, Villringer A and Einhaupl KM (1990) Angiotensin II induces endothelium-dependent vasodilation of rat cerebral arterioles. *Am J Physiol* 258:H1840-H1845.
- Haber RL, Decker JW and Einhaupl KM (1991) Angiotensin degradation products mediate endothelium-dependent dilation of rabbit brain arterioles. *Circ Res* 68: 1651-1657.
- Hageman RW, Moyer JS, Bachman ES, Sibony M, Magyar JL, Walsh JE, Smithies O, Kregg JJ and O'Brien DA (1998) Angiotensin-converting enzyme and male fertility. *Proc Natl Acad Sci USA* 95:2659-2667.
- Hall KM, Hansworth JM, Ball AE, Folgenhauer GF, Hoshik H and Harding JW (1993) Identification and characterization of a novel angiotensin binding site in cultured vascular smooth muscle cells that is specific for the angiotensin (3-8) peptide. *Regul Pept* 50:117-119.
- Hall KM, Venkateswarren S, Hansworth JM, Schelling ME and Harding JW (1996) Characterization of a functional angiotensin IV receptor on murine microvascular endothelial cells. *Regul Pept* 68:107-115.
- Hansen MJ, Coffman TM, Lashua A, Koide M, Zile MR, Oliverio ML, DeLuette G, Cooper G and Caron MG (1998) Pressure-overload hypertrophy is unaltered in mice devoid of AT₁ receptors. *Am J Physiol* 274:H959-H973.
- Han HM, Shimizu SI, Kanashiro CA, Oliveira I, Han SW and Palva AC (1998) Residue Val254, His256, and Phe269 of the angiotensin II AT₁ receptor are not involved in ligand binding but participate in signal transduction. *Mol Endocrinol* 12:810-814.
- Henda RS, Krebs LT, Harding JW and Henda RS (1998) The angiotensin IV-AT₁ receptor system in the rat kidney. *Am J Physiol* 274:F390-F399.
- Hessmann S, Stosch MP, Krebs LT, Hall KM and Harding JW (1993) Elucidation of a specific binding site for angiotensin II (3-8), angiotensin IV, in mammalian heart membranes. *J Pharmacol Exp Ther* 268:1038-1042.
- Hall KM, Krebs LT, Miller-Wing AY, Cohen J, Wright JW and Harding JW (1993) The angiotensin II (3-8) peptide binds to a specific binding site in guinea pig hippocampus. *Brain Res* 653:340-343.
- Henderson J and Folks D (1997) The effects of the aminopeptidase inhibitors amastatin and bestatin on angiotensin-avoid neuronal activity in brain. *Brain Research* 424:299-304.
- Harding JW, Imboden H and Folks D (1996) Is angiotensin II the centrally active form of angiotensin? *Experientia* 42:705-709.
- Harding JW, Wright JW, Swanson GM, Hansworth JM and Krebs LT (1994) AT₁ receptors: Specificity and distribution. *Kidney Int* 46:310-312.
- Hauger RL, Aguilera G, Bunkal A and Catt KJ (1983) Characterization of angiotensin II receptors in the anterior pituitary gland. *Mol Cell Endocrinol* 25:203-212.
- Hayashida W, Horiuchi M and Druze VJ (1998) Intracellular third loop domain of angiotensin II type 2 receptor: Role in mediating signal transduction and cellular function. *J Biol Chem* 273:11955-11959.
- Healy DP, MQ and Tzouyounakis M (1995) Localization of angiotensin II type 1 receptor subtype mRNA in rat pituitary. *Am J Physiol* 268:F220-F225.
- Hennaker FMJ and Saverio JM (1995) Quantitative autoradiography of angiotensin II AT₁ receptors with [¹²⁵I]OPC 42112. *Brain Res* 671:39-38.
- Hirth SL (1998) Genetic deletion and overexpression of angiotensin II receptors. *J Med* 78:756-768.
- Hein L, Bank GS, Pratt RE, Druze VJ and Kobilka BK (1995a) Behavioral and cardiovascular effects of lurasidone, a novel AT₁ type 2 receptor gene in mice. *Nature (Lond)* 377:744-747.
- Hein L, Druze VJ and Bank GS (1995b) Linkage mapping of the testicular angiotensin II receptor and its subtypes in mice. *Genomics* 30:639-671.
- Hill-Kaparakos N, Kaparakos ME, Rickard RB, Patel JG, Mainelli T, Madson KM and Tisher OC (1999) Angiotensin II-stimulated nitric oxide release from porcine pulmonary endothelium is mediated by angiotensin IV. *J Am Soc Nephrol* 10:631-641.
- Hirai K, Song H, Kaneshara H, Shinta N, Ueda H, Kim S, Miyasaka H, Katsukawa Y and Miyasaka M (1998) Pituitary-dependent expression of the testicular angiotensin II receptor and its subtypes in rats. *Endocrinology* 131:177-185.
- Hiruma Y, Inoue A, Hirose B and Igarashi H (1997) Angiotensin II stimulates the proliferation of osteoblast-like populations of cells from rat calvariae. *Biochem Biophys Res Commun* 230:175-178.
- Hirth SL, Schanbye HT, Greenlee JW and Schwartz TW (1994) Identification of peptide binding residues in the extracellular domains of the AT₁ receptor. *J Biol Chem* 269:30953-30959.
- Hobbs S, Blum A, LeRoux C, Culman J and Unger T (1995) Angiotensin receptors in the brain. *Pharmacol Toxicol* 77:805-815.
- Hobbs S, Culman J, Bosse M, Grolli T and Unger T (1996) Effect of angiotensin AT₁ and muscarinic receptor blockade on renin-angiotensin system and vasopressin release. *Eur J Pharmacol* 300:119-123.
- Enkhbakhsh G, Schmidt-Schweda SL, Kozar A, Duls J, Finkbe B, Raf N, Fesli R, Hansson G and Jander G (1994) Functional significance of angiotensin receptors in the heart. *Eur Heart J* 15 (Suppl) D38-31.
- Holmström J, Graf K, Winkler M and Fleck End Regitz-Zakowicz V (1997) Molecular biology of the renin-angiotensin system. *Am J Physiol* 273:H655-H664.
- Homma Y, Sakamoto H, Taniguchi M, Aoki M, Takemura T and Ooyama T (1993) Evidence for involvement of phospholipase C-gamma 2 in signal transduction of

- platelet-derived growth factor in vascular smooth-muscle cells. *Biochem J* 290: 649-655.
- Hong RK, Rhee BY, Shin YW and Yoo SR (1994) Characterization of PD-2198 and OPG41210 induce unmasking of low concentration effects of angiotensin II in rabbit abdominal aorta. *J Pharmacol Exp Ther* 271:1591-1596.
- Horiuchi M, Akaboshi M and Duan WJ (1999) Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. *Hypertension* 33:618-621.
- Horiuchi M, Hayashida H, Akaboshi M, Tamura K, Davies L, Lehtonen JT and Duan WJ (1998) Stimulation of angiotensin II receptor, AT₁ and AT₂ receptors, regulates STAT activation by negative cross-talk. *Circ Res* 84:678-682.
- Horiuchi M, Hayashida H, Kambe T, Yamada T and Duan WJ (1997a) Angiotensin type 2 receptor dephosphorylates Bcl2 by activating tyrosine-activated protein kinase phosphatase-1 and induces apoptosis. *J Biol Chem* 272:19092-19098.
- Horiuchi M, Kikita G, Yasuda T, Miyayama M, Nakajima M and Duan WJ (1995) The growth dependent expression of angiotensin II type 2 receptor is regulated by transcription factor interferon: regulatory factor-1 and -2. *J Biol Chem* 270:20225-20230.
- Horiuchi M, Yamada T, Hayashida H and Duan WJ (1997b) Interferon: regulatory factor -2 up-regulates angiotensin II type 2 receptor and induces apoptosis. *J Biol Chem* 272:11952-11958.
- Huang XG, Richards EM and Summers C (1996) Mitogen activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. *J Biol Chem* 271:15635-15641.
- Huckle WR and Eary HS (1994) Regulation of cell proliferation and growth by angiotensin II. *Prog Growth Factor Res* 6:177-184.
- Humphrey PPA and Barnard EA (1998) International Union of Pharmacology. XIX. The IUPHAR receptor code: A proposal for an alphanumeric classification system. *Pharmacol Rev* 50:871-900.
- Humphrey PPA, Spedding M and Vanheste P (1994) Receptor classification and nomenclature. The revolution and the resolution. *Trends Pharmacol Sci* 15:208-209.
- Hunyady L (1999) Molecular mechanisms of angiotensin II receptor internalization. *J Am Soc Nephrol* 10:847-858.
- Hunyady L, Balla T and Catt KJ (1996a) The ligand binding site of the AT₁ receptor. *Trends Pharmacol Sci* 17:196-197.
- Hunyady L, Baulieu AA, Balla T and Catt KJ (1996b) Independence of type I angiotensin II receptor endocytosis from G protein coupling and signal transduction. *J Biol Chem* 271:29202-29206.
- Hunyady L, Bor M, Balla T and Catt KJ (1994b) Identification of a cytoplasmic serine-threonine motif that determines angiotensin-induced internalization of the AT₁ angiotensin receptor. *J Biol Chem* 269:33593-33598.
- Hunyady L, Bor M, Balla T and Catt KJ (1994a) A conserved NPFLPY sequence contributes to angiotensin binding and signal transduction but is not an internalization signal for the AT₁ angiotensin receptor. *J Biol Chem* 269:15908-15916.
- Hunyady L, Bor M, Balla T and Catt KJ (1995) Critical role of a conserved intramembrane tyrosine residue in angiotensin II receptor activation. *J Biol Chem* 270:7028-7036.
- Hunyady L, Li H, Jagadeesh G, Zhang M, Gaborit Z, Mihailic B and Catt KJ (1998) Dependence of AT₁ angiotensin receptor function on adjacent asparagine residues in the seventh transmembrane helix. *Mol Pharmacol* 64:427-434.
- Hunyady L, Zhang M, Jagadeesh G, Bor M, Balla T and Catt KJ (1998b) Dependence of agonist activation on a conserved asparagine residue in the third intracellular loop of the AT₁ angiotensin receptor. *Proc Natl Acad Sci USA* 95:10040-10045.
- Hewlett A, van Rossum G, Watanawa M and Palschert J (1998) Angiotensin II stimulation of the stress-activated protein kinase in small mesangial cells is mediated by the angiotensin AT₁ receptor subtype. *Eur J Pharmacol* 343:297-302.
- Ichiki T, Herold GL, Kambayashi Y, Burdick S and Isangami T (1994) Cloning of the cDNA and the genomic DNA of the mouse angiotensin II type 2 receptor. *Biochim Biophys Acta* 1218:60-64.
- Ichiki T and Isangami T (1995a) Depression genomic organization and transcription of the mouse angiotensin II type 2 receptor gene. *Circ Res* 76:733-736.
- Ichiki T and Isangami T (1995b) Transcriptional regulation of the mouse angiotensin II type 2 receptor gene. *Hypertension* 33:720-725.
- Ichiki T, Kambayashi Y and Isangami T (1996) Differential nitric oxide of angiotensin II AT₁ receptor between SHR and WKY vascular smooth muscle cells. *Kidney Int* 49 (Suppl 65):14-17.
- Ichiki T, Kambayashi Y and Isangami T (1996a) Multiple growth factors modulate mRNA expression of angiotensin II type 2 receptor in R373 cells. *Circ Res* 79: 1070-1076.
- Ichiki T, Lalobay PA, Shiota G, Okuyama S, Imagawa Y, Fogo A, Nishimura T, Ichikawa I, Hogan BL and Isangami T (1996b) Effects on blood pressure and reduced reproductive behavior in mice lacking angiotensin II type 2 receptor. *Nature Lond* 377:148-150.
- Ichiki T, Ueki M, Kato M, Funakoshi Y, Ito K, Egashira K and Takedaishi A (1998) Downregulation of angiotensin II type 1 receptor gene transcription by nitric oxide. *Hypertension* 31:342-348.
- Isangami T, Guo DF and Kikuchi Y (1994) Molecular biology of angiotensin II receptors. Overview. *J Hypertens* 12 (Suppl 1):93-104.
- Isoe Y, Nakamura N and Griendling KK (1997) A review of mutagenesis studies of angiotensin II type 1 receptor, the three-dimensional receptor model in search of the agonist and antagonist binding sites and the hypothesis of a receptor activation mechanism. *J Hypertens* 15:703-714.
- Jahida M, Jahida T, Thomas SM and Bark BC (1998) Activation of extracellular signal-regulated kinases (ERK1/2) by angiotensin II is dependent on Src in vascular smooth muscle cells. *Circ Res* 82:7-12.
- Jahizaka N, Griendling KK, Lasseigne B and Alexander RW (1998) Angiotensin II type 1 receptor: Relationship with caveolae and caveolin after initial agonist stimulation. *Hypertension* 32:464-469.
- Jirani A, Stambrook C, Dauter R, Garzido ML, Torres M and Saverdva JM (1999) Angiotensin II receptor subtypes and phosphoinositide hydrolysis in rat adrenal medulla. *Endocrinology* 135:4641-4646.
- Laszaki K, Shigetani Y and Fujimoto M (1993) Molecular cloning and characterization of the angiotensin receptor subtype in porcine smooth muscle. *Eur J Pharmacol* 216:1-10.
- Ito M, Oliverio MI, Macman PJ, Best CF, Maeda N, Smithies O and Coffman TM (1998) Regulation of blood pressure by the type 1_A angiotensin II receptor gene. *Proc Natl Acad Sci USA* 95:3521-3525.
- Janick P, Pillan A, Prost JT and Vilaine JP (1992) Role of angiotensin subtype 2 receptor in acetylcholine formation after vascular injury. *Hypertension* 20:737-745.
- Jarvis MF, Gensler GW and Lam CK (1992) The angiotensin receptors 1-5: 8 fragment potentially inhibits [125I] angiotensin II binding to non-AT₁ or -AT₂ recognition sites in bovine adrenal cortex. *Eur J Pharmacol* 210:319-322.
- Jayadev S, Smith TJ, Jagadeesh G, Baulieu AA, Hunyady L and Catt KJ (1999) N-linked glycosylation is required for optimal AT₁ angiotensin receptor expression in C6 glioblastoma cells. *Endocrinology* 140:2010-2017.
- Jeeva D, Ochedalski T, Kiss A and Aguilera G (1998) Brain angiotensin II modulates sympathetic and hypothalamic pituitary adrenocortical activation during stress. *J Neuroendocrinol* 10:67-72.
- Ji R, Leung M, Zhang YJ, Catt KJ and Sandberg K (1994) Differential structural requirements for specific binding of nonpeptide and peptide antagonists to the AT₁ angiotensin receptor. *J Biol Chem* 269:16533-16536.
- Ji E, Sandberg K, Zhang Y and Catt KJ (1993) Molecular cloning, sequencing and functional expression of an amphibian angiotensin II receptor. *Biochem Biophys Res Commun* 194:716-722.
- Ji H, Zhang W, Zhang Y, Catt KJ and Sandberg K (1995) Genetic transfer of a nonpeptide binding site to a previously unresponsive angiotensin II receptor. *Proc Natl Acad Sci USA* 92:9240-9244.
- Johnson AK and Gross PM (1983) Sensory circumventricular organs and brain homeostatic pathways. *FASEB J* 7:678-686.
- Johnson MC and Aguilera G (1987) Angiotensin II receptor subtypes and coupling to signaling systems in cultured fetal fibroblasts. *Endocrinology* 120:1366-1374.
- Johren O, Imboden H, Hauser W, Maye I, Sanvitto GL and Saverdva JM (1997a) Localization of angiotensin-converting enzyme, angiotensin II, angiotensin II receptor subtypes, and vasotensin in the mouse hypothalamus. *Brain Res* 773:215-227.
- Johren O, Isangami T and Saverdva JM (1998) AT₁, AT₂, and AT₃ angiotensin II receptor subtype gene expression in the rat brain. *Endocrinology* 133:332-339.
- Johren O, Isangami T and Saverdva JM (1996) Localization of AT₁ angiotensin II receptor gene expression in rat brain by *in situ* hybridization histochemistry. *Mol Brain Res* 37:191-199.
- Johren O, Sanvitto GL, Egidio G and Saverdva JM (1997b) Angiotensin II AT_{1A} receptor mRNA expression is induced by estrogen-progesterone in dopaminergic neurons of the female rat arcuate nucleus. *J Neurosci* 17:6313-6321.
- Johren O, Mordt B, Himmelfarb G, Maye I and Saverdva JM (1995a) A computer modeling predicted mechanism for angiotensin II receptor activation. *J Protein Chem* 14:381-398.
- Joseph MT, Maigret B and Saverdva JM (1995b) Proposals for the angiotensin II receptor-bound conformation by comparative computer modeling of AII and cyclic analogs. *Int J Pept Protein Res* 46:515-528.
- Kai H, Alexander RW, Uchida-Pak M, Lyons PR, Akers M and Griendling KK (1996) G-Protein binding domains of the angiotensin II AT_{1A} receptors mapped with synthetic peptides selected from the receptor sequence. *Biochem J* 324:715-727.
- Kakimaru K, Feroia M, Tawfikian J, Karguzi J, Kiselevskiy M, Syvonen AJ, Vardanian D, Peltonen I and Kontala K (1999) Evidence for involvement of the type 1 angiotensin II receptor locus in essential hypertension. *Hypertension* 33: 844-849.
- Kajstura J, Cigola B, Malinova A, Li P, Cheng W, Menges LG and Anversa P (1997) Angiotensin II induces apoptosis of adult ventricular myocytes *in vitro*. *J Mol Cell Cardiol* 29:658-670.
- Kakar SS, Sellers JC, Devor DC, Muggave LC and Neill JD (1992) Angiotensin II type 1 receptor subtypes (AII_{1A}) differential tissue expression and hormonal regulation. *Biochem Biophys Res Commun* 311:1090-1096.
- Kakimaru J, Ichiki T, Kiyama S, Hogan BL, Fogo A, Isangami T and Ichikawa I (1995) Developmental expression of renal angiotensin receptor gene in the mouse. *Kidney Int* 47:140-147.
- Kambayashi Y, Burdick S and Isangami T (1993a) Peptide growth factors markedly decrease ligand binding of angiotensin II type 2 receptor in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 194:478-482.
- Kambayashi Y, Richter S, Takahashi K, Tsuchi S, Itoh H, Hamakubo T and Isangami T (1993b) Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J Biol Chem* 268:24543-24546.
- Kambayashi Y, Nagata K, Ichiki T and Isangami T (1996) Insulin-like growth factor-induced expression of angiotensin type 2 receptor in vascular smooth muscle cells. *Eur J Biochem* 229:558-565.
- Kang J, Isangami T and Pomeroy P (1994) Angiotensin II type 2 receptor stimulation of neuronal K⁺ currents involves an inhibitory GTP binding protein. *Am J Physiol* 267:C1389-C1397.
- Kang J, Richards EM, Pomeroy P and Summers G (1995) Modulation of the delayed rectifier K⁺ current in neurons by an angiotensin II type 2 receptor fragment. *Am J Physiol* 268:C278-C282.
- Kang J, Summers C and Pomeroy P (1992) Modulation of net outward ionic current in cultured neurons by angiotensin II. Involvement of AT₁ and AT₂ receptors. *Brain Res* 580:337-344.
- Kang J, Summers C and Pomeroy P (1993) Angiotensin II type 2 receptor-mediated K⁺ currents in cultured neurons. *Am J Physiol* 265:C307-C315.
- Karim SS, Hussain A and Gresser R (1996) Angiotensin II receptor subtypes and non-peptide binding to the AT₁ receptor. *Clin Exp Pharmacol Physiol* (Suppl) S558-S562.
- Kelley JA, Björk PA, Hodges JC and Taylor DO Jr (1992) Renal hemodynamic and

- of cultured embryonic remodulatory interstitial cells through the AT₂ receptor. *Kidney Int* 53:89-98.
- Mario J and Jurd S (1983) Angiotensin II inhibits adenylate cyclase from adrenal cortex glomerular source. *FEBS Lett* 159:97-101.
- Mario J, Maignan B, Joseph-Bourcier P, Nouet S, Lombard C and Benoitou JG (1994) Tyr295 in the seventh transmembrane domain of the AT₁ angiotensin II receptor is essential for its coupling to phospholipase C. *J Biol Chem* 269:20616-20618.
- Merrero MB, Paxton WG, Duff JL, Berk BC and Bernstein KE (1994) Angiotensin II stimulates tyrosine phosphorylation of phospholipase C- γ 1 in vascular smooth muscle cells. *J Biol Chem* 269:10931-10935.
- Merrero MB, Schiffer B, Li B, Sun J, Hary JB and Ling BN (1997) Role of Janus kinase/signal transducer activator of transcription and mitogen-activated protein kinase cascades in angiotensin II- and platelet-derived growth factor-induced vascular smooth muscle cell proliferation. *J Biol Chem* 272:24584-24590.
- Merrero MB, Schiffer B and Paxton WG (1985) Direct stimulation of JAK/STAT pathway by the angiotensin II AT₁ receptor. *Nature (London)* 317:647-650.
- Merrero MB, Vassara MJ, Jo H, Hary JB and Paxton WG (1998) Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: Role of SHP-1 and SHP-1. *Am J Physiol* 276:C1216-C1223.
- Marshall GR, Yim W and Needleman P (1970) A specific competitive inhibitor of angiotensin II. *Proc Natl Acad Sci USA* 67:1624-1628.
- Martin J and Gervantes C (1995) Angiotensin converting enzyme inhibitors and the role of nitric oxide and excitatory amino acids in improvement of cognition and memory. *J Auton Pharmacol* 15:129-149.
- Martin MM, Su B and Elton TS (1994) Molecular cloning of the human angiotensin II type-2 receptor. *Genomics Biophys Res Commun* 205:645-651.
- Masaki H, Kurahara T, Yanaka A, Itohara N, Nawa Y, Mori Y, Murawasa S, Kizume K, Murayama K, Horuchi M, Dasa YJ, Takahashi H, Iwasaki T, Inada M and Murawasa F (1988) Characteristic expression of angiotensin II AT₁ receptor across strains of mice: Involvement of AT₁ receptor-substantiated and cholinergic effects. *J Clin Invest* 101:527-533.
- Matsukawa JM, Bigan G, Zhou N and Morris GJ (1990) ¹²⁵I-NMR studies of [Sar¹] angiotensin II conjugation by nuclear Overhauser effect spectroscopy in the reacting frame (Roe) of the aromatic ring in dimethylsulfoxide. *Peptides* 11:359-366.
- Matsukawa JM, Easwari J, Karamida M, Marmostouides T, Malvarinos A, Yonaghi R, Wu Q and Morris GJ (1990) Role of the N¹-terminal domain of angiotensin II (AngII) and [Sar¹] angiotensin II on conformation and activity. NMR evidence for aromatic vinyl clustering and peptide backbone folding compared with [Ile¹,2,3] angiotensin II. *Chem Commun* 1989:5309-5312.
- Matsubara H (1993) Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res* 83:1189-1191.
- Matsubara H, Kanasaki M, Murawasa S, Tachibana Y, Nio Y and Inada M (1994) Differential gene expression of angiotensin II receptor subtypes in rat cardiac fibroblasts and cardiomyocytes in culture. *J Clin Invest* 98:1589-1601.
- Matsukawa T, Nishimura H, Usumoto H, Kakeuchi J, Niuma F, Inagami T, Pagan A and Ishikawa I (1990) Constitutive missplicing/regional targeted deletion of the angiotensin type 1a receptor gene. Evidence against the role for local angiotensin in the in vivo feedback regulation of renin synthesis in juxtaglomerular cells. *J Clin Invest* 95:1877-1877.
- Matsukawa T and Ishikawa I (1997) Biological functions of angiotensin and its receptors. *Annu Rev Physiol* 59:395-412.
- Maturana AD, Bunney MJ, Capponi AM, Vallotton MB and Rossier MF (1999a) Angiotensin II type 1 receptor activation modulates L- and T-type calcium channel activity through distinct mechanisms in bovine adrenal glomerular cells. *J Recept Sign Transduct Res* 19:509-520.
- Maturana AD, Cami AJ, Demaree N, Vallotton MB, Capponi AM and Rossier MF (1999b) Angiotensin II negatively modulates L-type calcium channels through a pertussis toxin-sensitive G protein in adrenal glomerular cells. *J Biol Chem* 274:19458-19464.
- Mausy CA, Hwang O, Rigloff AM, Wu Li and Chung TP (1992) Cloning, expression, and characterization of a gene encoding the human angiotensin II type 1A receptor. *Biochem Biophys Res Commun* 186:971-974.
- McClellan KJ and Bellver JA (1998) *Encyclopedia. Drugs* 66:713-718.
- McGinley MJ, Bicknell RJ, Davis D, McAllan RM, Vinals I, Weisinger RS and Oldfield BJ (1992) Effluent neural pathways of the lamina terminalis subserving nociception. *Prog Brain Res* 91:397-402.
- McGinley MJ and Oldfield BJ (1998) The brain as an endocrine target for peptide hormones. *Trends Neuroendocrinol* 9:349-353.
- McWhinney CR, Hunt RA, Central KM, Dostal DE and Baker KM (1997) The type 1 angiotensin II receptor couples to Stat1 and Stat3 activation through Jak2 kinase in neonatal rat cardiac myocytes. *J Mol Cell Cardiol* 29:2515-2524.
- McKaffy B, Stoll M, Stachurski EP and Unger T (1990) The angiotensin AT₁ receptor inhibits proliferation and promotes differentiation in PC12W cells. *Mol Cell Biochem* 125:287-297.
- Mendelsohn FA, Qian H, Rasmussen JM, Aguilera G and Catt KJ (1984) Autoradiographic localization of angiotensin II receptors in rat brain. *Proc Natl Acad Sci USA* 81:1878-1879.
- Menzel MM, Mufson JE, Wainer BH and Levy AI (1983) Central cholinergic pathways in the rat: An overview based on an alternative nomenclature (Ch1-Ch5). *Neuroscience* 10:1185-1201.
- Metschies KP, Stoll M, Goltjes P, Fou M and Unger T (1992) Angiotensin II is antiproliferative for coronary endothelial cells in vitro. *Pharm Pharmacol Lett* 2:150-152.
- Millan MA, Carrault P, Izant S, Zemel S, Catt KJ and Aguilera G (1989) Novel sites of expression of Angiotensin II type-1 receptors: In the late gestation fetus. *Science (Wash DC)* 244:1330-1332.
- Millan MA, Jacolowitz DM, Aguilera G and Catt KJ (1991) Differential distribution of AT₁ and AT₂ angiotensin II receptors in the rat brain during development. *Proc Natl Acad Sci USA* 88:11440-11444.
- Millan MA, Jacolowitz DM, Catt KJ and Aguilera G (1989) Distribution of angiotensin II receptors in the brain of subhuman primates. *Peptides* 11:243-253.
- Millan LA, Abdel-Rahman EM and Siragy HM (1989) Angiotensin II and nitric oxide: A question of balance. *Regul Pept* 31:1-10.
- Miller-Wang AY, Sasaworth JM, Sandiana MP, Wright JW, Bepth RK, Grove XL and Harding JW (1993) Central angiotensin IV receptors: Distribution and specificity in primate pig brain. *J Pharmacol Exp Ther* 266:1718-1728.
- Miyata S, Fuku YH, Hasegawa A and Kamei S (1989a) Role of aromaticity of agonist switch of angiotensin II in the activation of the AT₁ receptor. *J Biol Chem* 264:7303-7310.
- Miyata SI and Kamei SK (1990b) Angiotensin II type 1 and type 2 receptors bind angiotensin II through different types of epitope recognition. *J Hypertens* 17:397-401.
- Minner R, Blag B and Keyser J (1995) Losartan-sensitive AT₁ receptors linked to β -adrenergic-dependent cardiac secretion through a novel signaling pathway. *J Biol Chem* 270:20842-20851.
- Moeller I, Allen AM, Choi SY, Zhuo J and Mendelsohn PA (1998) Bioactive angiotensin peptides. *J Nutr Biochem* 12:289-293.
- Moeller I, Choi S, Oldfield BJ, McKinley MJ, Casley D and Mendelsohn PA (1995) Localization of angiotensin II binding sites to motor and sensory neurons in the sheep spinal cord and hindbrain. *Brain Res* 701:301-305.
- Moeller I, Law RA, Mendelsohn PA, Smith AJ, Brennan MS, Tetra TJ and Choi SY (1997) The fragment LVV-hemaphysin-1 is an endogenous ligand for the AT₂ receptor in the brain. *J Neurochem* 68:2350-2357.
- Moeller I, Pacione G, Mendelsohn PA, Adren GP, Casley D and Choi SY (1996a) Distribution of AT₂ receptors in the Macaca fascicularis brain. *Brain Res* 712:307-334.
- Moeller I, Small DH, Reed G, Harding JW, Mendelsohn PA and Choi SY (1996b) Angiotensin II inhibits neurite outgrowth in cultured embryonic chicken sympathetic neurons. *Brain Res* 728:61-66.
- Monk JB, Williams RJ, Bagdasarian W, Thibault SJ and Williamson JR (1980) Angiotensin II effects on the cytosolic free Ca²⁺ concentration in N1E-115 neuroblastoma cells: Kinetic properties of the Ca²⁺ transient measured in single Purkinje cells. *J Neurochem* 34:271-287.
- Morales C, Bihoreau C, Chouin S, Currow KM, Corvol P and Clauser E (1996) Polar residues in the transmembrane domains of the type 1 angiotensin II receptor are required for binding and coupling. Reconstitution of the binding site by co-expression of two deficient mutants. *J Biol Chem* 271:15077-1513.
- Monnot C, Weber V, Sztanek J, Bihoreau C, Teatich B, Corvol P and Clauser E (1991) Cloning and functional characterization of a novel mast-cell-associated gene, modulating intracellular angiotensin II stimulation. *Mol Endocrinol* 5:1477-1487.
- Morera C, Rao-Ramjanahary R, Audin V, Nivon C and Enjalbal A (1994) Angiotensin II effects on second messengers involved in prolactin secretion are mediated by AT₁ receptor in anterior pituitary cells. *Mol Cell Neurosci* 8:597-603.
- Morimoto M, Matsuda H, Mori Y, Murawasa S, Masaki H, Murayama K, Tsutsumi Y, Shibauchi Y, Tanaka Y, Nakajima T, Oda K and Iwasaki T (1999) Angiotensin II-induced transactivation of β -adrenergic growth factor receptor regulates fibroblast and transforming growth factor- β synthesis via transcriptional and posttranscriptional mechanisms. *Circ Res* 84:1077-1084.
- Morimoto S and Ogihara T (1995) TCY-110 A new angiotensin II type-1 receptor antagonist. *Cardiovasc Drug Res* 12:133-144.
- Morivichi R, Shibata S, Himeeno A, Jaber G, Hoe KL and Saavedra JM (1998) Molecular cloning and pharmacological characterization of an atypical β -opioid angiotensin II type-1 receptor and its mRNA expression in brain and peripheral tissues. *Brain Res Mol Brain Res* 60:234-246.
- Morrell NW, Upton PD, Kotchen S, Zuzely A, Yacoub MH, Polak JM and Wharton J (1989) Angiotensin II activates MAPK and stimulates growth of human pulmonary artery smooth muscle via AT₁ receptors. *Am J Physiol* 277:L440-L448.
- Morris ML, Li P, Callahan MP, Oliverio MJ, Coffman TM, Bosch SM and Dils D (1999) Neuroendocrine effects of dehydronin in mice lacking the angiotensin AT_{1A} receptor. *Hypertension* 34:692-695.
- Mosimann R, Imboden I and Felix D (1990) The neuronal role of angiotensin II in thirst, sodium appetite, excretion and memory. *Biol Res Camb Philo Soc* 71:545-551.
- Mrug M, Stopka T, Julian BA, Prehnl JP and Prehnl JP (1997) Angiotensin II stimulates proliferation of normal early erythroid progenitors. *J Clin Invest* 100:2310-2314.
- Mukherjee A, Kulkarni P, McMahon SM and Negro-Villar A (1982) Evidence for the presence and characterization of angiotensin II receptors in rat anterior pituitary membranes. *Hypertension* 10:666-667.
- Mukoyama M, Tachibana M, Horuchi M, Sasegawa H, Pratt RS and Dasa YJ (1993) Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven transmembrane receptors. *J Biol Chem* 268:24591-24594.
- Muramatsu H and Gounis AS (1999) Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension* 27:760-765.
- Murawasa S, Matsubara H, Uchikawa M and Inada M (1983) Regulatory elements within the 5' flanking region of the gene for the angiotensin II type 1a receptor for the rat. *J Biol Chem* 258:25998-26003.
- Murphy TJ, Alexander RW, Griendling KK, Runge MB and Bernstein KE (1991) Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature (London)* 16:233-236.
- Murphy TJ, Nakamura Y, Takeuchi K and Alexander RW (1993) A cloned angiotensin II receptor identified from the turkey oviduct is pharmacologically distinct from mammalian angiotensin receptors. *Mol Pharmacol* 44:1-7.
- Nahmias G, Carabuzzi SM, Brien-Suizen M, Lazari D, Villainen P and Struwing AD (1995) Angiotensin II AT₁ receptors are functionally coupled to tyrosine phosphorylation in N1E-115 neuroblastoma cells. *Biochem J* 306:87-92.
- Nahmias G and Struwing AD (1993) The angiotensin AT₁ receptor: Searching for signal-transduction pathway and physiological function. *Trends Pharmacol Sci* 14:229-235.
- Najamati C, Abdulnag NG and Ridge KP (1998) Light-induced exposure of the

- cytoplasmic end of transmembrane helix seen in rhodopsin. *Biochemistry* 36: 12494-12499.
- Narita M, Hatanaka HG, Fujisawa M, Hayashida W, Morioka T, Zhang L, Taniuchi M, Pratt R and Drazu JV (1990) The angiotensin II (AT₂) receptor antagonizes the growth effects of the AT₁ receptor: Genin-of-function study using gene transfer. *Proc Natl Acad Sci USA* 87:10683-10687.
- Nakajima M, Makiyama M, Pratt RS, Hatanaka M and Drazu JV (1993) Cloning of cDNA and analysis of the gene for mouse angiotensin II type 2 receptor. *Biochem Biophys Res Commun* 197:938-939.
- Narazawa R, Lantieri L, Xu L and Nadel J (1994) Role of specific isoforms of protein kinase C in angiotensin II and L-phenylephrine action in rat adrenal glomerular cells. *Mol Cell Endocrinol* 101:69-86.
- Niwerl L, Stenbom C and Sauerwald J (1994a) Angiotensin II AT₂ receptor stimulation induces the upper limit of cerebral blood flow autoregulation: Agonist effects of CGP 42112 and PD123119. *J Cereb Blood Flow Metab* 14:38-44.
- Niwerl L, Stenbom C and Sauerwald J (1994b) Angiotensin II AT₂ receptor stimulation increases cerebrovascular resistance during hemorrhagic hypotension in rats. *Regul Pept* 52:21-29.
- Niwerl BG and Tonks NK (1997) Protein tyrosine phosphatases in signal transduction. *Curr Opin Cell Biol* 9:189-200.
- Nickerson G, Baumer AT, Grohe C, Kahlert S, Strehlow K, Rosenkranz S, Steinhilber A, Beckers P, Smith GJ, Deenen MJ, Vetter M and Böhm M (1996) Estrogen modulates AT₁ receptor gene expression in vitro and in vivo. *Circulation* 97:2187-2201.
- Nickerson G, Leebs U, Schnabel P, Knorr PA and Böhm MP (1997) Down-regulation of aortic and cardiac AT₁ receptor gene expression in transgenic *tgAren-2* rat rats. *Br J Pharmacol* 121:124-130.
- Nickerson G, Röhling J, Strehlow K, Schnabel P and Böhm M (1998) Insulin induces upregulation of vascular AT₁ receptor gene expression by posttranscriptional mechanisms. *Circulation* 98:549-560.
- Nishi K and Badoer E (1997) Excitation of sympathetic neurosecretory cells by angiotensin II. *Nature New Biol* 388:173-174.
- Nitamaru P, Laboulaye P, Kameichi Y, Okubo S, Yoshida T, Ishida A, Nishida A, Page A, Inagami T, Hogen B and Ishiura H (1995) Gene targeting in mice reveals a requirement for angiotensin in the development and maintenance of kidney morphology and growth factor regulation. *J Clin Invest* 96:2947-2954.
- Nikolovska-Gjy and Marshall GR (1993) Three-dimensional recognition requirements for angiotensin analogs: A novel solution for an old problem. *Biochem Biophys Res Commun* 196:222-228.
- Nikolovska-Gjy, Kallayevich CV, Kallayevich K, Zhang WJ and Marshall GR (1994) Conformational analysis of two cyclic analogs of angiotensin: Implications for the biologically active conformation. *Biochemistry* 33:3591-3598.
- Nio Y, Matsubara H, Murasawa S, Kanazawa M and Inada M (1986) Regulation of gene transcription by angiotensin II receptor subtypes in myocardial infarction. *J Clin Invest* 95:40-45.
- Nishimura H, Yonekura S, Hohenfeller K, Miyazaki Y, Ma J, Hunsley TE, Yoshida H, Ishida T, Tsuruguchi T, Hogen B, Page A, Brock JW 3rd, Inagami T and Ishiura H (1990) Role of the angiotensin type 2 receptor gene in congenital anomalies of the kidney and urinary tract, CAKUT, of mice and man. *Mol Cell* 3:1-10.
- Noda K, Peng YH, Liu XP, Sood Y, Hussain A and Karnik SS (1996) The state of the AT₁ angiotensin receptor is generated by angiotensin II induction. *Biochemistry* 35:16458-16462.
- Noda K, Sood Y and Karnik SS (1996a) Interaction of Ph⁸ of angiotensin II with Lys²⁸⁶ of AT₁ receptor in agonist activation. *J Biol Chem* 270:28511-28514.
- Noda K, Sood Y, Kinoshiwa A, Boyle TP, Graham RM, Hussain A and Karnik SS (1996b) Tetraene and carboxylate groups of angiotensin receptor antagonists bind to the same subunit by different mechanisms. *J Biol Chem* 270:2484-2489.
- Novasman RD, Fung CC, Kory AD and Kadowitz PJ (1993) Analysis of responses to Ang IV: Effects of D₁₂-123119 and D₁₂-753 in the pulmonary circulation of the rat. *Am J Physiol* 265:L502-L503.
- Novasman S and Nishimura C (1991) Signal transduction from the angiotensin AT₁ receptor. *Trends Endocrinol Metab* 1:111-116.
- Novasman Y, Vancuro A, Oda N, Yamasaki Y, Matsura N, Yamada S, Inoue K, Kimura R, Suzuki F and Ishiura H (1993) Angiotensin II receptor subtypes in bovine and human ventricular myocytes. *J Pharmacol Exp Ther* 270:566-571.
- Obermüller N, Unger T, Culean J, Gohlke P, de Gasparo M and Botzaf SP (1991) Distribution of angiotensin II receptor subtypes in rat brain nuclei. *Neuro Sci Lett* 129:1-16.
- O'Brien RM and Granzer DK (1991) Regulation of gene expression by insulin. *Biochem J* 278:609-619.
- Okubo N, Matsubara H, Novasman Y, Mori Y, Murayama S, Kijima K, Maruyama K, Maeki E, Tsutsumi Y, Shibasaki Y, Iwasaki T and Inada M (1997) Angiotensin type 2 receptors are expressed by cardiac fibroblasts from failing myopathic hamster hearts and inhibit cell growth and fibroblast collagen metabolism. *Circulation* 96:3954-3958.
- Omishi J, Ishida M, Shibata T, Inagami T, Murekami K and Miyazaki K (1992) The rat angiotensin II AT₂ receptor couple with three different signal transduction pathways. *Biochem Biophys Res Commun* 186:1094-1101.
- Oyama K, Yamano Y, Sano T, Nakagami Y, Hanzawa K, Morikawa I and Inagami T (1995) Disulfide bridges in extracellular domains of angiotensin II receptor type 2. *Regul Pept* 67:141-144.
- Olinda M, Kawahara Y and Yokoyama M (1989) Angiotensin II type 1 receptor-mediated activation of Ras in cultured rat vascular smooth muscle cells. *Am J Physiol* 271:H206-H209.
- Okyu S, Inagami K, Kaneko T and Yamashita H (1987) Angiotensin II sensitive neurons in the suprachiasmatic, midbrain and anterior and posterior third ventricle of rats in vitro. *Brain Res* 413:137-147.
- Oldfield BJ, Badoer E, Harris DK and McKinley MD (1994) Fos production in retrogradely labeled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or angiotensin II. *Neuroscience* 60:255-262.
- Oliverio MI, Best CF, Kim HS, Arandehorst WJ, Smithies O and Coffman TM (1997) Angiotensin II responses in AT_{1A} receptor-deficient mice: A role for AT_{1B} receptors in blood pressure regulation. *Am J Physiol* 273:R1518-R1523.
- Oliverio MI, Kim HS, Ito M, Le T, Audoly L, Best CF, Hillier S, Kluckman K, Maeda N, Smithies O and Coffman TM (1998a) Reduced renal and systemic blood pressure regulation in mice lacking both type 1 and type 2 AT₁ receptors for angiotensin II. *Proc Natl Acad Sci USA* 95:15490-15491.
- Oliverio MI, Maessen K, Best CF, Ito M, Maeda N, Smithies O and Coffman TM (1998b) Renal growth and development in mice lacking AT_{1A} receptors for angiotensin II. *Am J Physiol* 274:R63-R70.
- Orth SR, Weinreich T, Bonaldi S, Weiss M and Ritz E (1995) Angiotensin II induces hypertrophy and hyperplasia in adult human mesangial cells. *Exp Nephrol* 32:33-38.
- Ozono R, Wang ZQ, Moore AF, Inagami T, Strang HM and Carey RM (1997) Expression of the Subtype 2 Angiotensin II (AT₂) Receptor Protein in Rat Kidney. *Hypertension* 30:1338-1342.
- Page HH (1990) Hypertension research. A memoir 1920-1990. *Hypertension* 16:189-200.
- Page HH and Halpern OM (1940) A crystalline spermac substance (angiotensin) resulting from the reaction between renin and renin substrate. *J Exp Med* 71:29-42.
- Palin DT, Denning GS Jr and Kaessner NE (1979) Historical development of saralasin. *Kidney Int* 16:87-910.
- Pat MG, Florio T and Störck PJ (1992) G-protein activation of a hormone-stimulated phosphatase in human tumor cells. *Science* 256:1215-1217.
- Parada F and Wormal M (1974) Dose-response curves for angiotensin II and synthetic analogues in three types of sarcoma tissues: Existence of different forms of receptor sites for angiotensin II. *Br J Pharmacol* 30:299-307.
- Parada F, Dalk-Toussaint N, Parada FW, Thibault R and Nemer M (2000) Overexpression and remodeling. *Proc Natl Acad Sci USA* 97:91-93.
- Paroz C, Bardin S, Misery-Lancelot S, Gratin D, Corvel P and Clausen E (2000) Identification of mutations that constitutively activate the angiotensin II type 1A receptor by screening a randomly mutated cDNA library with an original pharmacological bioassay. *Proc Natl Acad Sci USA* 97:616-620.
- Patel JM, Li YD, Zhang C, Griebel G, Badoer E, Kallayevich K and Black BK (1998) Increased expression of endothelin is linked to Ang IV-mediated activation of lung endothelial NOS. *Am J Physiol* 274:R1794-R1801.
- Patel JM, Martinez JR, Li YD, Zhang C, Badoer E, Kallayevich K and Black BK (1998) Increased expression of endothelin is linked to Ang IV-mediated activation of lung endothelial NOS. *Am J Physiol* 275:L1061-L1068.
- Pavet MD, Blaudet L, Drole P, Darrondo J, Guillen G and Gallo-Pavet N (1995) "K⁺-activated" K⁺ channels by angiotensin II in rat adrenal glomerular cells: Involvement of a G protein. *Mol Endocrinol* 9:935-947.
- Pavet MD (1997) Renin-angiotensin system: Biochemistry and mechanisms of action. *Physiol* 18:313-370.
- Pavet MD and Levens RN (1980) Molecular approaches to the study of angiotensin receptors. *Adv Exp Med Biol* 171:1-21.
- Pederson ES, Harding JW and Wright JW (1998) Attenuation of neoplasia-induced signal learning impairment by an Angiotensin IV analog. *Regul Pept* 74:97-103.
- Petit A, Geoffroy P and Bellais S (1998) Expression of angiotensin II type-1 receptor and phospholipase C α in the human placenta. *J Soc Gynecol Invest* 5:316-321.
- Phillips MI (1987) Functions of angiotensin II in the central nervous system. *Annu Rev Physiol* 49:413-435.
- Phillips MI, Wang H, Kimura B, Speth RC and Ghazal N (1997) Brain angiotensin and the female reproductive cycle. *Adv Exp Med Biol* 377:567-570.
- Phillips MI and Summers C (1995) Angiotensin II in central nervous system physiology. *Regul Pept* 78:1-10.
- Pieroni ME and Freer RJ (1992) Analysis of the active conformation of angiotensin II. A comparison of all and non-peptide AT₁ antagonists. *Pept Res* 5:102-125.
- Pieroni ME, Reiss K, Chung W, Cicala G, Kojima K, Nishikawa JA, Riek M, Copogrosso MC and Anversa P (1997) p38 mitogen-activated protein kinase in the regulation of the renin-angiotensin system. *Exp Cell Res* 234:487-505.
- Pohorik BF, Haworth EG and Garrison JO (1989) Role of N¹ in coupling angiotensin receptors to inhibition of adenylate cyclase in hepatocytes. *J Biol Chem* 264:1820-1829.
- Pohorik BF, Nordrup JK, Bauer PH, Fraser ED and Garrison JO (1991) Inhibitory GTP-binding regulatory protein G_i can couple angiotensin II receptors to inhibition of adenylate cyclase in hepatocytes. *Mol Pharmacol* 40:150-157.
- Potlitz M, Shih A, Richard DS, Christen L and Guilleme RT (1990) Effect of angiotensin on AT₁ receptor affinity for antagonist analogs of angiotensin II. *Recept Channels* 6:61-73.
- Pollman MJ, Yamada T, Horiechi M and Gibbons GH (1996) Vasoactive substances regulate vascular smooth muscle cell apoptosis: Countervailing influences of nitric oxide and angiotensin II. *Circ Res* 78:748-758.
- Puente AG, Hodge JC, Sen J, Bumpus FM and Hussain A (1991) Biochemical properties of the ovarian granulosa cell type 2 angiotensin II receptor. *Endocrinology* 128:1291-1295.
- Quian H, Pipolo L and Thomas WG (1990) Identification of protein kinase phosphorylation sites in the angiotensin II (AT₁) receptor. *Biochem J* 264:637-644.
- Quian H, Pipolo L, Ruz S, Munzel T, Turpe M, Freeman BA, Greenfield SR and Harrison DG (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NAD(P)H oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 97:619-629.
- Quian H, Kessler SP, Colmaneras C and Sen GO (1998) Selective restoration of renal fertility in mice lacking angiotensin converting system by sperm-specific expression of the testicular isozyme. *J Clin Invest* 101:1019-1023.
- Rouglet LB, Curcio-Neves C, Lora LS, Brasil Jr and Lopes AG (1999) Angiotensin II

- activates the protein-kinase Na^+ -ATPase from renal proximal tubules through a G-protein. *Biochem Biophys Res Commun* 141:639-643.
- Rao GN and Bieri JC (1993) Active oxygen species stimulate vascular smooth muscle cell growth and protein-angiotensin expression. *Circ Res* 70:535-539.
- Re RN, Vissard BJ, Brown J, LeCheney and Bryan SB (1984) Angiotensin II receptors in chromatin. *J Hypertens* 2(Suppl 2):S271-S273.
- Reagan LP, Flanagan LM, Yee DK, Ma LY, Sotnik RP and Fluharty SJ (1994) Immunohistochemical mapping of angiotensin type 2 (AT_2) receptors in rat brain. *Brain Res* 662:45-59.
- Reagan LP, Theveniaux M, Yang XD, Siemens IR, Yee DK, Reisine T and Fluharty SJ (1993a) Development of polyclonal antiserum against angiotensin type 2 receptors. *Proc Natl Acad Sci USA* 90:1515-1519.
- Reagan LP, Ye X, Muretschik CH and Fluharty SJ (1993b) Down-regulation of angiotensin II receptor subtypes and desensitization of cyclic GMP production in neuroblastoma N1E-115 cells. *J Neurochem* 60:24-31.
- Reagan LP, Ye XH, Mir R, DeFina LR and Fluharty SJ (1990) Up-regulation of angiotensin II receptors by *in vitro* differentiation of murine N1E-115 neuroblastoma cells. *Mol Pharmacol* 56:86-88.
- Renoux A, Pournier-Saladuk MC, Devic C, Zini S, Roques BP, Corvol P and Lorenzen-Cortes O (1995) Angiotensinase A inhibitors as potential central antihypertensive agents. *Proc Natl Acad Sci USA* 96:13415-13420.
- Regitz-Zagrosek V, Tiede N, Heymans A, Bauer T, Neuss M, Bohn A, Steffen C, Hildebrandt A, Hettler R and Fleck E (1995) Regulation chamber localization and myocyte distribution of angiotensin receptors in human hearts. *Circulation* 91: 1461-1471.
- Regitz-Zagrosek V, Neuss M, Holzmeister J, Wannecke C and Fleck E (1996) Molecular biology of angiotensin receptors and their role in human cardiovascular disease. *J Mol Med* 74:623-631.
- Richard DE, Christie JA, Caron M and Guillemeau G (1997a) Stimulation of the angiotensin II type 1 receptor on bovine adrenal glomerulosa cells activates a temperature-sensitive internalization-recycling pathway. *Mol Cell Endocrinol* 129:203-218.
- Richard DE, Laporte SA, Bernier SG, Leduc R and Guillemeau G (1997b) Desensitization of AT_1 receptor-mediated cellular responses requires long term receptor down-regulation in bovine adrenal glomerulosa cells. *Endocrinology* 138:3535-3536.
- Riedel WM, Ammer P and Habel RL (1995) Different mechanisms of L-arginine induced dilation of brain arteries in normotensive and hypertensive rats. *Brain Res* 671:21-26.
- Rittel W, Iselin B, Kuppeler H, Runkel B and Selwyser R (1957) Synthesis of a highly effective angiotensin II analog (L-asparagyl-L-argyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-phenylalanine) in Germany. *Wochschr Chem Abt* 40: 614-624.
- Roberts KA, Krebs LT, Kramer EA, Shaffer MJ, Harding JW and Wright JW (1995) Anterograde identification of brain angiotensin II binding sites and differential *c-fos* expression following intracerebroventricular injection of angiotensin II and IV in rats. *Brain Res* 68:13-21.
- Robinson OS, Crooks CB, Shinkman PG and Gallagher M (1990) Behavioral effects of MK-801 mimic deficits associated with hippocampal damage. *Psychobiology* 17:156-164.
- Rodríguez H, de Gasparo M, Grosfeld E, Stulez P, Burkert P, Eberhard M and Erpe P (1996) Angiotensin II-receptor subtypes in human atria and evidence for alterations in patients with cardiac dysfunction. *Eur Heart J* 17:1112-1120.
- Rodríguez H, de Gasparo M, Grosfeld E, Stulez P and Erpe P (1991) Identification of angiotensin II receptor subtypes on human Vainhofen. *Schweiz Med Wochenschr* 121:103.
- Rogg H, Schmidt A and de Gasparo M (1990) Identification and characterization of angiotensin II receptor subtypes in rabbit ventricular myocardium. *Biochem Biophys Res Commun* 174:416-422.
- Roffa A, Weber-Rodriguez J, Regitz-Zagrosek V, Kallisch H, Riedel K and Fleck E (1994) Genetic polymorphism of angiotensin II type 1 (AT_1) receptor gene. *Eur Heart J* 15 (Suppl D):102-112.
- Roman RJ and Crowley AW Jr (1986) Characterization of a new model for the study of pressure autoregulation in the rat. *Am J Physiol* 244:R190-R198.
- Rousselle JJ, McNeill N, Decker E, Melchior S, Ong H and DeLanau A (1990) Hemodynamic properties of the angiotensin II receptor from bovine adrenal zona glomerulosa. *Biochem J* 268:44-48.
- Roper D, Krüger P, Grotzinger J, Wollmer A and Stitzinger W (1995) Models of G-protein-coupled receptors revised for family-wide compliance with experimental data. A new sequence accommodation suggested for helix G. *Recept Channels* 3:57-67.
- Rowe BP, Grove DL, Bayliss DL and Speth RC (1980a) Angiotensin II receptor subtypes in the rat brain. *Eur J Pharmacol* 186:339-342.
- Rowe BP, Kallisch PW and Speth RC (1980b) Immunohistochemical localization of angiotensin II receptor 2 binding sites on noradrenergic neurons of the locus coeruleus of the rat. *J Neurochem* 35:558-560.
- Rowe BP, Bayliss DL and Speth RC (1982) Analysis of angiotensin II receptor subtypes in individual rat brain nuclei. *Neuroscience* 10:553-573.
- Ruan X, Wagner C, Castellaneta G, Kurts A and Arndorfer W (1997) Regulation of angiotensin II receptor AT_1 subtypes in renal afferent arterioles during chronic changes in sodium flow. *J Clin Invest* 100:1072-1081.
- Sauvage JM (1992) Brain and pituitary angiotensin. *Endocr Rev* 13:824-840.
- Sauvage JM, Vianenham M and Shigematsu K (1995) Localization of angiotensin AT_2 receptors in the rat heart conduction system. *Eur J Pharmacol* 235:301-303.
- Sabri A, Govindarajan G, Griffin TM, Byron RL, Sammel AM and Lucchesia PA (1995) Calcitonin and protein kinase C-dependent activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. *Circ Res* 83:861-871.
- Sadoshima J, Qin Z, Morgan JP and Izumo S (1995) Angiotensin II and other hypertrophic stimuli mediate by G-protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 30-kDa $\text{p}39$ kinase in cardiac myocytes. The critical role of Ca^{2+} -dependent signaling. *Circ Res* 76:1-15.
- Selman S, Ratkul A, Waters S, Humphreys FM and Catt KJ (1975) Competitive binding activity of angiotensin II analogues in an adrenal cortex radioligand-receptor assay. *Endocrinology* 97:276-282.
- Sandberg K (1994) Structural analysis and regulation of angiotensin II receptors. *Physiol Rev* 74:229-253.
- Sandberg K, Bor M, Ji H, Markwick A, Millan MA and Catt KJ (1990) Angiotensin II-induced calcium mobilization in oocytes by signal transduction gap junctions. *Science* (Wash DC) 249:293-301.
- Sandberg K, Ji H and Catt KJ (1991) Regulation of angiotensin II receptors in rat brain during dietary sodium changes. *Hypertension* 23:137-141.
- Sandberg K, Ji H, Clark AD, Stanina H and Catt KJ (1992a) Cloning and expression of a novel rat angiotensin II receptor. *J Biol Chem* 267:8465-8468.
- Sandberg K, Ji H, Luedt T and Catt KJ (1992b) Interreceptor communication between follicular AT_1 receptors and Xenopus laevis oocytes: Mediation by an inositol 1,4,5-trisphosphate-dependent mechanism. *Cel Biol* 41:167-167.
- Sandberg K, Ji H, Millan MA and Catt KJ (1991) Amphiphilic myocardial angiotensin II receptors are distinct from mammalian AT_1 and AT_2 receptors. *FEBS Lett* 284:281-284.
- Sandmann SL, Yu MH, Kaschuba S and Unger T (1999) Differential roles of AT_1 and AT_2 receptor subtypes in the expression of Na^+ - HCO_3^- -symporter and Na^+ - H^+ -exchanger in the rat heart after myocardial infarction. *Disch Med Wochenschr* 124 (Suppl 2):S287 (P71).
- Sano T, Ohyama K, Yamano Y, Nakagami Y, Nakazawa S, Kikyo M, Shirai H, Blank JS, Ertan JH and Isomura T (1997) A domain for G protein coupling in carboxyl-terminal tail of rat angiotensin II receptor type 1. *J Biol Chem* 272:25631-25636.
- Sardina M, Hansworth JM, Kreis LF and Harding JW (1993) AT_2 receptor binding characteristics: D-amino acid- and glycine-substituted peptides. *Peptides* 14:493-504.
- Sardina MF, Hansworth JM, Krishnan R and Harding JW (1994) AT_2 receptor structure-binding relationship: N-terminal modified angiotensin II analogs. *Peptides* 15:1389-1400.
- Sasaki H, Yamano Y, Sordani S, Iwai N, Murray JJ, Higashawa M, Matsuda Y and Isomura T (1991) Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type 1 receptor. *Nature (Lond)* 351:282-283.
- Seydewitz PJ, Al MS, Semmelits MD, Dean LN and Bernheim KE (1996) Angiotensin II signal transduction pathways. *Regul Pept* 67:1-10.
- Schambye JT, Hjorth SA, Bergema DJ, Sathie G and Schwartz TW (1994) Differentiation between binding sites for angiotensin II and nonpeptide antagonists on the angiotensin II type 1 receptor. *Proc Natl Acad Sci USA* 91:7046-7050.
- Scheuer DA and Perrenne MH (1993) Angiotensin type 2 receptors mediate depressor phases of biphasic pressure responses to angiotensin. *Am J Physiol* 264:R1977-R1983.
- Schiffman MT, Khoshdel MC and Ferrario CM (1995) *Angiotensin II-17*: Evidence for novel actions in the brain. *J Cardiovasc Pharmacol* 16 (Suppl 4):19-24.
- Schiffman MT, Paxton WG, Choi Q, Marero MB and Bernstein KB (1995) Angiotensin II controls $\text{p}42$ activity via $\text{p}42$ kinase. *J Biol Chem* 270:10329-10333.
- Schmitt S, Beige J, Wille-Friedel M, Micale MC, Sharma AM and Ritz E (1997) A polymorphism in the gene for the angiotensin II type 1 receptor is not associated with hypertension. *J Hypertens* 15:1385-1388.
- Schmitt U and Birk KJ (1997) Angiotensin II signal transduction. Stimulation of multiple mitogen-activated protein kinase pathways. *Trends Endocrinol Metab* 8:261-266.
- Schmieder JB, Freyner T, Yang T, Huang YG, Oliverio MI, Coffman T and Briggs AP (1997) Absence of tubuloglomerular feedback responses in AT_1 receptor-deficient mice. *Am J Physiol* 273:F916-F920.
- Schorf W, Booz WG, Dotla DE, Conrad KM, Chang KC and Baker KM (1993) Angiotensin II is autocrine in neonatal rat cardiac fibroblasts. *Circ Res* 72:1245-1254.
- Schultz LH (1997) Polymorphism of the angiotensin-converting enzyme gene and cardiovascular disease. *J Mol Med* 76:767-775.
- Soni AJ, Chang RS, Lett VJ and Siegel FK (1992) Cardiac angiotensin receptors: Effects of selective angiotensin II receptor antagonists Dp 753 and P213217 in rabbit heart. *J Pharmacol Ther* 261:837-855.
- Sotti LA, Griffin CA, DiCicco-Belli E, Lorenzen-Cortes C, Corvol P and Schambelan M (1996) Tissue-specific regulation of type 1 angiotensin II receptor mRNA levels in the rat. *Hypertension* 28:403-403.
- Sotti LA, Grady EP, Griffin CA, Kallisch PW and Schambelan M (1992a) Distribution of angiotensin II receptor subtypes in rat and human kidney. *Am J Physiol* 262:F258-F264.
- Sotti LA, Griffin CA, Grady EP, Kallisch PW and Schambelan M (1992b) Characterization of angiotensin II receptor subtypes in rat heart. *Circ Res* 71:452-459.
- Simple PF, Boyd AS, Davies PM and Morton JF (1976) Angiotensin II and its heptapeptide (2-8) heptapeptide (3-8) and pentapeptide (4-8). Metabolites in arterial and venous blood of man. *Circ Res* 39:671-678.
- Sm L, Hull HG and Soffer RJ (1984) Isolation of an angiotensin II-binding protein from bovine liver. *Proc Natl Acad Sci USA* 81:1679-1683.
- Sorrento G, Dudley PE, Eberhard R and Guillemeau G (1990) The marked disparity between the sizes of angiotensin type 2 receptors from different tissues is related to different degree of N-glycosylation. *Mol Pharmacol* 45:1112-1118.
- Sorrento G, Dudley PE, Eberhard R and Guillemeau G (1990) Analysis of the role of N-glycosylation in cell surface expression and binding properties of angiotensin II type 2 rat pheochromocytoma cells. *Biochem J* 271:997-1004.
- Shanmugasu S, Lemke GA, Gane JMR, Corvol P and Larent-Cortes CM (1995) Cloning of the angiotensin II type 2 (AT_2) receptor mRNA in the rat. *Kidney Int* 47:1095-1100.
- Shanmugasu S, Monnot G, Corvol P and Gane JC (1994) Distribution of type 1 and type 2 angiotensin receptor subtypes messenger RNAs in the rat fetus. *Hypertension* 23:137-151.
- Shapiro MS, Wulmsuth LP and Hills B (1994) Angiotensin II inhibits calcium and M^+ current channels in rat sympathetic neurons via G protein. *Neuron* 12:1313-1320.

- Sharp M, Pettes D, Brozier G, Clark A, Peters J, Fleming S and Mullins J (1996) Targeted inactivation of the *Ang II* gene in mice. *Hypertension* 28:1126–1131.
- Shibata T, Suzuki O, Chikada K, Murakami K and Miyazaki H (1996) Identification of regions in the human angiotensin II receptor type 1 responsible for G_q and G₁₂ coupling by mutagenesis study. *Biochem Biophys Res Commun* 218:383–389.
- Shinkai T and Oota H (1996) Effect of angiotensin II on the proliferation of macrophages from the adult rat aortic intima in culture. *Peptides* 16:26–32.
- Shirai H, Takahashi K, Katsuta T and Inagami T (1995) Mapping of G protein coupling sites of the angiotensin II type 1 receptor. *Hypertension* 25(4 Pt 4):726–730.
- Siemens IR, Adler HJ, Adyiah Mh SJ and Fluharty SJ (1991) Biochemical identification of subunitized angiotensin II receptors from murine neuroblastoma N1E-115 cells by covalent cross-linking and radiolabel purification. *J Biol Chem* 266:171–176.
- Sirois JR, Reagen LP, Yu DK and Fluharty SJ (1994) Biochemical characterization of two distinct angiotensin II receptor populations in murine neuroblastoma N1E-115 cells. *J Neurochem* 62:1108–1116.
- Slemmon IR, Yee DK, Reagen LP and Fluharty SJ (1994) Affinity purification of angiotensin type 2 receptors from N1E-115 cells: Evidence for agonist-induced formation of multimeric complexes. *J Neurochem* 62:857–864.
- Slone G, Aubry K and Douglas AG (1992) Angiotensin II-induced changes in guanine nucleotide binding and regulatory proteins. *Hypertension* 19:148–162.
- Stragy HM and Carey RM (1996) The subtype-2 (AT₂) angiotensin receptor regulates renal cyclic guanosine 3',5'-monophosphate and AT₁ receptor mediated prostaglandin E₂ production in conscious rats. *J Clin Invest* 97:1978–1983.
- Stragy HM and Carey RM (1997) The subtype 2 (AT₂) angiotensin receptor mediates renal production of cyclic nucleotides in conscious rats. *J Clin Invest* 100:284–289.
- Stragy HM and Carey RM (1997) The subtype 3 angiotensin receptor regulates renal prostaglandin F₂-alpha formation in conscious rats. *Am J Physiol* 274: R1103–R1107.
- Stragy HM and Carey RM (1998) Protective role of the angiotensin AT₂ receptor in a renal wet hypertension model. *Hypertension* 31:1237–1242.
- Stragy HM, de Gasparo M and Carey RM (2000) Angiotensin type 2 receptor mediated vasopressin-induced hypertension in conscious rats. *Hypertension* 35:1074–1077.
- Stragy HM, Inagami T, Ishii K and Carey RM (1996) Sustained hypotension by angiotensin II and its mechanism in mice lacking the subtype-2 (AT₂) angiotensin receptor. *Proc Natl Acad Sci USA* 93:6506–6510.
- Strobel I, Mossessli L, Calverley MJ, Scholze C, Crestel A and Tremblay B (1999) Effect of valsartan, a type-1 angiotensin II receptor (AT₁) antagonist, on PAI-1 accumulation in smooth muscle cells. *Thromb Haemostasis* 81:A486.
- Stroger LT, Leuts K, Kahn CR, Scholze C, Crestel A and Tremblay B (1999) The amino acid sequence of hypertension II. *J Exp Med* 194:183–197.
- Slone LW, Wong HC, Sternini G, Grady EP, Bunnett NW and Walsh JH (1994) The conserved NPK₁ motif present in the putative-regulating peptide receptor is a general sequence requirement for G-protein coupling. *Hypertension* 23:1770–1772.
- Smith RD (1998) Marked loss dependence of ¹²⁵I-Angiotensin II binding to atypical sites on *Mycoplasma hyarum*. *Peptides* 19:165–169.
- Smith RD, Bunkel AJ, Dent P and Cusi JC (1990) Raf-1 kinase activation by angiotensin II in adrenal glomerulosa cells: Roles of G_q, phosphatidylinositol 3-kinase, and Ca²⁺ influx. *Endocrinology* 124:1386–1391.
- Smith RD, Hampel L, Oliveira-Royce A, Chalkik B, Jayaram S and Catt KJ (1998) Angiotensin-induced phosphorylation of the angiotensin AT₁ receptor is localized to a serine/threonine-rich region of its cytoplasmic tail. *Mol Pharmacol* 64:835–841.
- Snyder EM and Breddi DS (1991) Nucleic acids as a neuronal membrane. *Trends Pharmacol Sci* 12:102–115.
- Song K, Allen AM, Paxinos G and Mendelsohn FA (1993) Mapping of angiotensin II receptor subtype heterogeneity in rat brain. *J Comp Neurol* 316:467–484.
- Speth RC and Kim KM (1990) Discrimination of two angiotensin II receptor subtypes with a selective agonist analogue of angiotensin II-pamphosphinylalanine¹ angiotensin II. *Biochem Biophys Res Commun* 169:997–1000.
- Speth RC, Rowe BP, Grove KL, Carter MC and Seyler D (1991) Sulphydryl reducing agents distinguish two subtypes of angiotensin II receptors in the rat brain. *Brain Res* 548:1–8.
- Shanabek JP, Choudhry M, Yukio E and Douchet CT (1994) Re-evaluation of the effects of putative cognitive disruptors in the reinforced-alternation T-maze task in the rat. *Br J Pharmacol* 112:155.
- Steckling UM, Bottari SP, Stoll M, Wagner J and Unger T (1998) Repression of c-fos and c-jun gene expression is not part of AT₁ receptor coupled signal transduction. *J Mol Med* 76:202–207.
- Steele MK, Negro-Vilar A and McLean SM (1981) Effect of angiotensin II on in vivo and in vitro release of anterior pituitary hormones in the female rat. *Endocrinology* 109:893–899.
- Stoll M, Steckling UM, Paul M, Bottari SP, Metzger R and Unger T (1995) The angiotensin AT₁ receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Exp Med* 182:651–657.
- Strobel M, Krueger NK, Thal T, Tang M and Salto H (1990) Distinct functional roles of the two intracellular phosphatase kinase domains of the receptor-linked protein tyrosine phosphatase LCA. *J Biol Chem* 265:2499–2507.
- Strodi U, Meffert S, Galliani S and Unger T (1998) Angiotensin II and NGF differentially influence microtubule proteins in PC12W cells—Role of the AT₁ receptor. *Mol Brain Res* 59:22–27.
- Strodi U, Blum A, Meleis R and Unger T (2000) Angiotensin AT₁ receptor stimulates ERK1 and ERK2 in quiescent but inhibits ERK1 in NGF-stimulated PC12W cells. *Mol Brain Res*, in press.
- Su B, Martin MM, Benson KB, Miller PJ and Elton TS (1994) The genomic organization and functional analysis of the promoter for the human angiotensin II type 1 receptor. *Biochem Biophys Res Commun* 204:1039–1045.
- Sugaya T, Nishimatsu S, Taniuchi T, Takizawa E, Yamaguchi T, Imamura K, Goto S, Imatani K, Hasegawa Y, Otsuka A, Uchida H, Segura M, Fukuta K, Fukumizu A and Murakami K (1995) Angiotensin II type 1 receptor-deficient mice with hypertension and hyperreninemia. *J Biol Chem* 270:18719–18723.
- Sugura N, Hagiwara H and Hirose S (1993) Molecular cloning of porcine soluble angiotensin-binding protein. *J Biol Chem* 268:18057–18072.
- Suzuki M (1991) Angiotensin II decreases cGMP levels in neuronal cultures from rat brain. *Am J Physiol* 260:C79–C87.
- Summers C and Phillips MT (1983) Central injection of angiotensin II alters catecholamine activity in the rat brain. *Am J Physiol* 244:R257–R263.
- Suzuki M, Tang W, Zelenski B and Rafraim MC (1991) Angiotensin II receptor subtypes and coupled with distinct signal-transduction mechanisms in neurons and astrocytes from rat brain. *Proc Natl Acad Sci USA* 88:7667–7671.
- Suzuki M, Matsuda H, Urakami M and Iwata M (1993) Rat angiotensin II (Type 1A) receptor mRNA regulation and subtype expression in myocardial growth and hypertrophy. *Circ Res* 73:458–467.
- Svensson L, Hansson GB, Jansson MF, Coleman JF, Wright JW, Hall KL, Miller-Wing AV, Stohs JW, Cook VL, Harding EO and Harding JW (1992) Discovery of a distinct binding site for angiotensin II (3–8), a putative angiotensin II receptor. *Regul Pept* 40:409–419.
- Szombathy T, Szalai O, Katalin B, Fallois T, Romics L and Csaszar A (1998) Association of angiotensin II type 1 receptor polymorphism with resistant essential hypertension. *Clin Chim Acta* 269:91–100.
- Takahashi T, Taniguchi T, Kunoike H, Kikkawa Y, Ishikawa Y and Yokoyama M (1995) Activation of Akt/protein kinase B after stimulation with angiotensin II in ventral smooth muscle cells. *Am J Physiol* 278:H1027–H1034.
- Tanaka M, Katsuya T, Rabeig H, Goto N, Nakata Y, Kamitani A, Mihai T, Elgadi J and Ogihara T (1998) Angiotensin II type 1 receptor gene polymorphism is associated with increases of left ventricular mass but not with hypertension. *Am J Hypertens* 10:116–121.
- Takayangi R, Ohnaka K, Sakai Y, Ikuyama S and Nawata H (1994) Molecular cloning and characterization of the promoter for human type 1 angiotensin II receptor. *Biochem Biophys Res Commun* 200:126–130.
- Telenchi K, Alexander RW, Nakamura Y, Tsujino T and Murphy TJ (1983) Molecular structure and transcriptional function of the rat vasomycin AT₁ angiotensin receptor. *Circ Res* 73:612–621.
- Teng H, Kuhn Y and Ogas J (1992) Effects of angiotensin and atrial natriuretic peptide on the cerebral circulation. *J Cereb Blood Flow Metab* 12:318–325.
- Tanaka M, Ohtsuki J, Onawa Y, Sugimoto M, Goto S, Naruse M, Murakami K and Miyazaki H (1994) Characterization of angiotensin II receptor type 2 during differentiation and apoptosis of rat ovarian cultured granulosa cells. *Biochem Biophys Res Commun* 207:559–563.
- Tanaka M, Matsuda S, Imai T, Fujii N, Miyazaki H, Ichikawa T, Naruse M and Inagami T (1998) Vascular response to angiotensin II is segregated through an integration of AT₁ receptor in AT₂ knockout mice. *Biochem Biophys Res Commun* 248:104–108.
- Tang H, Guo DP, Porter JP, Wanaka Y and Inagami T (1998) Role of cytoplasmic tail of the type 1A angiotensin II receptor in agonist- and phorbol ester-induced activation. *Circ Res* 82:620–627.
- Tang SS, Rogg H, Schumacher RM and Drenth VJ (1992) Characterization of nuclear angiotensin II-binding sites in rat liver and comparison with plasma membrane receptors. *Endocrinology* 131:874–880.
- Taniguchi K, Sugiyama Y, Goto Y, Ishida J, Takimoto M, Yagami K, Fukumizu A and Murakami K (1994) Angiotensinogen-deficient mice with hypertension. *J Biol Chem* 269:13384–13397.
- Tasawa S, Nakano T and Chiba S (1999) Angiotensin II type 1 receptor blockade prevents up-regulation of angiotensin II type 1A receptors in rat injured artery. *J Pharmacol Exp Ther* 288:889–904.
- Tsao DS, Der Sarkisian S, Hanes P and Deibel D (1989) Apoptotic and antiproliferative role of angiotensin II receptor subtype 2 in the heart of spontaneous hypertensive rats in vivo. *Hypertension* 23:836.
- Thekkumkara TJ, Thomas WG, Metel TJ and Baker KM (1998) Functional role for angiotensin II receptor (AT₁) 3'-untranslated region in determining cellular responses to agonist: Evidence for recognition by RNA binding proteins. *Biochem J* 326:255–264.
- Thomas WG (1999) Regulation of angiotensin II type 1 (AT₁) receptor function. *Regul Pept* 79:9–23.
- Thomas WG, Baker KM, Booz GW and Thekkumkara TJ (1998) Evidence against a role for protein kinase C in the regulation of the angiotensin II (AT₁) receptor. *Br J Pharmacol* 226:111–123.
- Thomas WG, Baker KM, Metel TJ and Thekkumkara TJ (1998) Angiotensin II type 1 receptor has two distinct regions of the cytoplasmic tail. A role for residues on the hydrophobic face of a putative amphipathic helix. *J Biol Chem* 273:21515–21525.
- Thomas WG, Metel TJ, Kule EC, Karoor V and Baker KM (1999) Phosphorylation of the angiotensin II (AT₁) receptor carboxyl terminus: A role in receptor endocytosis. *Mol Endocrinol* 12:1210–1224.
- Thomas WG, Qian H, Chang CS and Karoor S (2000) Agonist-induced phosphorylation of the angiotensin II (AT₁) receptor requires generation of a conformation that is distinct from the inositol phosphate- ϵ -signaling state. *J Biol Chem* 275:2893–2900.
- Thompson BV, Wade SM, Harrison AG, Salazar MC and Neubig RR (1998) Cotransfection of second and third intracellular loop fragments inhibit angiotensin AT₁ receptor activation of phospholipase C in HEK-293 cells. *J Pharmacol Exp Ther* 284:1222–1229.
- Tietzmann PA, Knebel P, Schmidt A, Harder S and Riebrock N (1996) Influence of the angiotensin antagonist valsartan on left ventricular hypertrophy in patients with essential hypertension. *Circulation* 94:1049–1054.
- Tian Y, Bunkel AJ, Bunkel AJ and Catt KJ (1995) Growth responses to angiotensin II in bovine adrenal glomerulosa cells. *Am J Physiol* 268:R335–R344.
- Tian Y, Bunkel AJ, Sandberg K, Barakat GC, Bunkel AJ and Catt KJ (1996) Properties of AT₁ and AT₂ angiotensin receptors expressed in adrenocortical Y-1 cells. *Am J Physiol* 270:R281–R288.
- Tigertsd R and Bergman PG (1968) Niere und Kreislauf. *Scand Arch Physiol* 8:263–271.

- Timmermans PMW, Wong PC, Chao AT, Herblin WP, Corrali DJ, Lee RJ, Wieders RM, Saye JAM and Smith JD (1985) Angiotensin II receptor and angiotensin II receptor antagonists. *Pharmacol Rev* 45:205-251.
- Tirel L, Bonnardaud A, Poirier G, Hecard S, Marquand-Vidal P, Evans A, Arveller P, Lee G, Kue Y, Duchesne P, Soulier P and Cambien F (1994) Spargic effects of angiotensin-converting enzyme and angiotensin II type 1 receptor gene polymorphism on risk of myocardial infarction. *Lancet* 344:910-913.
- Tsai P, Riviere DF, Tsai S, Inagami T, Lewan G, Sapiro J and Supiger C (1995) Localization of the genes encoding the three rat angiotensin II receptors, Agtr1a, Agtr1b, Agtr2, and the human AGTR2 receptor respectively to rat chromosomes 17q12, 2q24 and Xq24, and the human Xq24. *Cytogenet Cell Genet* 71:77-80.
- Trump-Kalmeier S, Hoflack J, Bruneval A and Hilbert M (1992) Modeling of G-protein-coupled receptors: Application to opiate, adrenaline, serotonin, acetylcholine, and mammalian opiate receptors. *J Med Chem* 35:5448-5462.
- Tsuchida S, Matsunaka T, Chen X, Ochiai S, Nishimura F, Nishimura H, Fogo A, Umemura H, Inagami T and Ichikawa I (1998) Murine double null mutants of the angiotensin type 1A and 1B receptor gene duplicates reveal abnormal phenotypes of angiotensinogen nullmice. *J Clin Invest* 101:755-760.
- Tsutsui K and Sawada JM (1982) Heterogeneity of angiotensin II AT₁ receptors in the rat brain. *Mol Pharmacol* 41:280-287.
- Tsutsui K, Strömberg C, Vasanthaiah M and Sawada JM (1991) Angiotensin II receptor subtypes in fetal tissues of rat-autoradiography, guanine nucleotide sensitivity and association with phospholipase hydrolysis. *Endocrinology* 129:1076-1082.
- Tsutsui K, Zorad S and Sawada JM (1993b) The AT₂ subtype of the angiotensin II receptors has differential sensitivity to dihalothal in specific brain nuclei of young rats. *Eur J Pharmacol Mol Pharmacol* 248:169-178.
- Tsutsui Y, Matsubara H, Masaki H, Kurihara H, Maruyama S, Takai S, Miyazaki M, Nozawa T, Ozono R, Nakagawa K, Miwa T, Kawada N, Mori Y, Shibasaki Y, Tanaka Y, Fujisawa Y, Koyama A, Takahashi H and Iwawata T (1990) Angiotensin II type 2 receptor: overexpression activates the vascular kinase system and causes vasodilation. *J Clin Invest* 104:925-935.
- Tsutsui Y, Matsubara H, Ohkubo N, Mori Y, Nozawa Y, Maruyama S, Kijima K, Maruyama H, Masaki H, Saito Y, Koyama A, Takahashi H, Iwawata T and Iwawata T (1988) Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. *Circ Res* 63:1038-1048.
- Tsuzuki S, Regazzi S and Inagami T (1996a) Inhibition of cell proliferation and activation of protein tyrosine phosphatases mediated by angiotensin II type 2 (AT₂) receptor in B279 rat fibroblasts. *Regul Pept* 68:385-390.
- Tsuzuki S, Ishii T, Nakakubo H, Kitami Y, Guo DF, Shirai H and Inagami T (1994) Molecular cloning and expression of the gene encoding human angiotensin II type 2 receptor. *Biochem Biophys Res Commun* 200:1449-1454.
- Tsuzuki S, Matsuo T, Regazzi S and Inagami T (1995) Angiotensin II type 2 receptor inhibits cell proliferation and activates tyrosine phosphatase. *Hypertension* 28:910-918.
- Tufo-McDonald A, Harrison AL, Everett A and Gomez R (1993) Ontogeny of type 1 angiotensin II receptor gene expression in the rat. *J Clin Invest* 91:530-537.
- Tufo-McDonald AL, Tuomano J, Harris L, Furber L and Gomez R (1995) Angiotensin II regulates nephrogenesis and renal vascular development. *Am J Physiol* 268:F130-F135.
- Unverdorff DA, Strader CD, Rivers R, Patchett AA and Prendergast K (1994) Structural model of angiotensin and angiotensin binding to the angiotensin II, AT₁ subtype, G protein coupled receptor. *Chem Biol* 1:213-221.
- Unger T (1990) The angiotensin type 2 receptor: Variations on an enigmatic theme. *J Hypertens* 17:715-726.
- Unger T, Chung O, Ciolino T, Oulman J, Calhoun S, Gohlke P, Hühls S, Meffert S, Stahl M, Struth U and Zhu Y (1998) Angiotensin receptors. *J Hypertens* 16 (Suppl 3):95-103.
- Ureña H, Healy B, Stewart RW, Burmpy FT and Haesler A (1989) Angiotensin II receptors in normal and failing human hearts. *J Clin Endocrinol Metab* 69:65-66.
- Ureña H, Nishimura H and Gantzy D (1990) Chymase-dependent angiotensin II forming system in humans. *Am J Physiol* 9:277-284.
- Ushio-Fukai M, Alexander RW, Alamo M and Griending KK (1998) p38 mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathway activated by angiotensin II. *J Biol Chem* 273:15022-15029.
- Ushio-Fukai M, Alexander RW, Akers M, Lyons PK, Lawrence G and Griending KK (1996a) Angiotensin receptor coupling to phospholipase D is mediated by the botanoma subunit of heterotrimeric G protein in vascular smooth muscle cells. *Mol Pharmacol* 66:49-149.
- Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Welch K and Griending KK (1996b) Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 271:22899-22904.
- Van Bilen M (1997) Signal transduction revisited: Recent developments in angiotensin II signalling. *Regul Pept* 71:101-109.
- Van Geel PF, Pinto VM, Bulckaen H and van Gier WH (1998) Is the A1160C polymorphism of the angiotensin II type 1 receptor involved in cardiovascular disease? *Eur Heart J* 19:573-578.
- Van Venter CAM, Vanheerden TAA, Lemons JML, Saxena PR, Schieckamp MADH and Danner AJH (1997) Angiotensin II mediated growth and antiproliferative effects in cultured neonatal rat cardiac myocytes and fibroblasts. *J Mol Cell Cardiol* 29:21-27.
- Venema RJ, Ju H, Venema VJ, Schelleffs B, Harp JB, Ling BN, Eaton DC and Marverio MB (1998a) Angiotensin II-induced association of phospholipase C γ with the G-protein-coupled AT₁ receptor. *J Biol Chem* 273:7703-7708.
- Venema RJ, Venema VJ, Eaton DC and Marverio MB (1998b) Angiotensin II-induced tyrosine phosphorylation of signal transducers and activators of transcription 1 is regulated by down-regulation of the tyrosine kinase and mitogen-activated protein kinase phosphatase 1. *J Biol Chem* 273:30755-30800.
- Vianello R, Clausen R, Corvol P and Mounier C (1998) Functional interactions of L-162,313 with angiotensin II receptor subtypes and mutants. *Eur J Pharmacol* 344:118-118.
- Villarroel JA, Kim NK, Ungch DG, Printz MP and Dillman WH (1993) Identification of functional angiotensin II receptors on rat cardiac fibroblast. *Circulation* 88:2849-2861.
- Vivianathan M, de Oliveira AM, Corro FMA and Saavedra JM (1994a) Expression of angiotensin II (AGP) 4212 binding site in healing wounds of the rat brain. *Brain Res* 685:285-290.
- Vivianathan M and Saavedra JM (1993) Expression of angiotensin II AT₂ receptors in the rat striatum: an experimental study of the effects of angiotensin II. *Brain Res* 685:285-290.
- Vivianathan M, Seltzer A and Saavedra JM (1994b) Heterogeneous expression of angiotensin II AT₁ receptor in neonatal rat carotid artery and aorta after balloon catheter injury. *Peptides* 15:1015-1022.
- Vivianathan M, Tsutsui K, Corro FMA and Saavedra JM (1991) Changes in expression of angiotensin receptor subtypes in the rat aorta during development. *Biochem Biophys Res Commun* 176:1561-1567.
- Voisin L, Larose L and Malbois S (1999) Angiotensin II stimulates serine phosphorylation of the adapter protein Nck: Physical association with the serine/threonine kinase Pak1 and cdc42. *J Biol Chem* 274:2517-2523.
- Wagner J, Gollan P, Ciochanawicz A and Ellis S (1999) Angiotensin II receptor type 1 gene expression in human glomerulonephritis and diabetes mellitus. *J Am Soc Nephrol* 10:545-551.
- Walshak G, Honnig V, Fischer T, Lindshak O, Hartmann B, Juppner A, Bauy E, Nissel R, Vitar R, Neichel D, Dudenhausen VJ, Haller H and Lohr FO (1999) Patients with preclampsia develop angiotensin antibodies against the angiotensin AT₁ receptor. *J Clin Invest* 103:945-953.
- Wang D, Jayadev S and Kacelnick JA (1998) Identification of a domain in the angiotensin II type 1 receptor determining G α coupling by the use of receptor chimera. *J Biol Chem* 273:16877-16883.
- Wang D, Jayadev S and Kacelnick JA (1999) Nitric oxide inhibits angiotensin II-induced activation of the calcium-sensitive tyrosine kinase protein-tyrosine kinase 2 without affecting epidermal growth factor receptor transactivation. *J Biol Chem* 274:24549-24555.
- Wang L, Eberhard M and Erne P (1996) Stimulation of DNA and RNA synthesis in cultured rabbit cardiac fibroblasts by angiotensin IV. *Clin Sci (Lond)* 88:507-508.
- Wang W and Gershengorn MC (1998) Rat TRH receptor type 2 exhibits ligand: basal state. *J Biol Chem* 273:16877-16883.
- Wang X and Murphy TJ (1998) Inhibition of cyclic AMP-dependent kinase by expression of a protein kinase inhibitor-enhanced green fluorescent protein fusion. *Proc Natl Acad Sci USA* 95:14849-14854.
- Wang X and Murphy TJ (1999) Angiotensin II-induced type 1 AT₁ receptor mRNA down-regulation in vascular smooth muscle cells. *Mol Pharmacol* 54:514-524.
- Wang X, Nickbarg G and Murphy TJ (1997) The vascular smooth muscle type 1 angiotensin II receptor mRNA is destabilized by cyclic AMP-elevating agents. *Mol Pharmacol* 52:781-787.
- Wayner MJ, Armstrong DL, Polan-Curtain JL and Denny JB (1993) Ethanol and diazepam inhibition of hippocampal LTP is mediated by angiotensin II and AT₁ receptors. *Peptides* 14:441-444.
- Wayner MJ, Oso T and Naley D (1973) Effects of angiotensin II on central neurons. *Pharmacol Biochem Behav* 1:679-691.
- Webb M, Liu CK, Cohen JB, Heisler A, Bogosian AE, Monshizadeh H, Molloy CG, Serafini R, Morland S, Murphy TJ and Dickinson MJ (1992) Molecular characterization of angiotensin II type II receptors in rat pheochromocytoma cells. *Peptides* 13:699-698.
- Weber H, Taylor DS and Molloy GJ (1994) Angiotensin II induces delayed mitogenesis and cellular proliferation in rat aortic smooth muscle cells. Correlation with expression of specific growth factors and reversal by saralasin. *J Clin Invest* 93:768-778.
- Weber KT and Brilla CG (1991) Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 83:1849-1860.
- Weber KT, Brilla CG, Campbell SE, Guarrta T, Zhu G and Sirovica K (1993) Myocardial fibrosis: Role of angiotensin II and aldosterone. *Basic Res Cardiol* 88 (Suppl 1):107-124.
- Weber KT, Sun Y and Campbell SE (1996a) Structural remodelling of the heart by fibrous tissue: Role of circulating hormones and local produced peptides. *Eur Heart J* 16 (Suppl N):12-18.
- Weber KT, Sun Y, Kottu LC and Clautens JPM (1996b) Connective tissue: a metabolic entity (Review). *J Mol Cell Cardiol* 27:197-220.
- Weber KT, Sun Y, Tyagi BC and Clautens JPM (1994) Collagen network of the myocardium: Function, structural remodelling and regulatory mechanisms. *J Mol Cell Cardiol* 27:279-292.
- Weerackody PR, Chatterjee PK, Mistary SK, McLane J, Hawksworth GM and McLay JS (1997) Selective antagonism of the AT₁ receptor inhibits the effect of angiotensin II on DNA and protein synthesis of rat proximal tubular cells. *Eur Nephrol* 5:253-262.
- Wenk H, Bigl V and Meyer U (1990) Cholinergic projects from mesencephalic nuclei innervate the rat heart: Evidence from retrograde tracing. *Brain Res* 525:305-316.
- Wen J (1993) Molecular basis of muscarinic acetylcholine receptor function. *Trends Pharmacol Sci* 14:309-313.
- Winters BJ, Morgan J, Rutherford RAD, Gattavadi JG, Cioerata A, Whitehead RP, De Leval MF, Vassiliou MT and Polak JM (1998) Differential distribution of angiotensin AT₁ receptors in the normal and failing human heart. *J Pharmacol Exp Ther* 284:332-338.
- Whitehead S, Mele M, Kamber B and De Gasparo M (1989) Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Commun* 164:284-291.
- Whitehead S, Mele M, Votari SP, Kamber B and De Gasparo M (1992) Radioiodinated CGP 4212: A novel highly affinity and highly selective ligand for the characterization of angiotensin AT₁ receptors. *Biochem Biophys Res Commun* 181:1665-1671.
- Wiesen W, Haul N, van Gool G, Nahr B, Ries U and Entzenstern M (1993) Pharma-

- ecological characterization of the novel nonpeptide angiotensin II receptor antagonist BIBP 277. *Br J Pharmacol* 110:246-252.
- Willart RT and Dzau VJ (1989) The renin-angiotensin system and experimental heart failure. *Circulation Res* 65:839-849.
- Woodcock EA and Johnson CI (1982) Inhibition of adenylate cyclase by angiotensin II in rat renal cortex. *Endocrinology* 111:1887-1891.
- Wright JW and Harding JW (1992) Regulatory role of brain angiotensins in the control of physiological and behavioral responses. *Brain Res Rev* 17:227-262.
- Wright JW and Harding JW (1994) Brain angiotensin receptor subtypes in the control of physiological and behavioral responses. *Neurosci Biobehav Rev* 18:61-83.
- Wright JW and Harding JW (1995) Brain angiotensin receptor subtypes AT₁, AT₂, and AT₃ and their functions. *Regul Pept* 59:289-305.
- Wright JW and Harding JW (1997) Important roles for angiotensin III and IV in the brain renin-angiotensin system. *Brain Res Rev* 28:59-124.
- Wright JW, Krebs LT, Stubb JW and Harding JW (1990) The angiotensin IV system: Functional implications. *Front Neuroendocrinol* 16:23-52.
- Wright JW, Munitan S, Murray GB, Amis HZ and Harding JW (1990) Aminopeptidase-induced elevations and reductions in blood pressure in the spontaneously hypertensive rat. *J Hypertens* 8:692-674.
- Wright JW, Sullivan MJ, Bredt CR, Hanesworth JW, Cushing LL and Harding JW (1987) Delayed carbonylterminal methylation of [¹²⁵I] angiotensins in the spontaneously hypertensive rat. *J Neurochem* 49:651-664.
- Wright JW, Sullivan MJ and Harding JW (1985) Dysfunction of central angiotensinergic aminopeptidase activity in spontaneously hypertensive rats. *Neurosci Lett* 61:351-356.
- Wyse B and Sernia C (1997) Growth hormone regulates AT₁-in angiotensin receptors in neutrophils. *Endocrinology* 138:4176-4180.
- Xu P, Graf K, Gesteira S, Flück B, Emswiler WA and Law RH (1999) Control role of the MAPK pathway in Ang II-mediated DNA synthesis and migration in rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 19:78-82.
- Xiong R and Marshall RC (1994) Angiotensin II depresses glutamate depolarizations and excitatory postsynaptic potentials in locus coeruleus through angiotensin II subtype 3 receptors. *Neuroscience* 62:163-175.
- Yamada H, Akiyama M, Loo M, Tamura K, David L, Lehtonen JYA, Dasa VJ and Horiuchi M (1999) AT₁ receptor and vascular smooth muscle cell differentiation in vascular development. *Hypertension* 33:1414-1419.
- Yamada T, Horiuchi M and Dasa VJ (1999) Angiotensin II type 2 receptor mediates programmed cell death. *Proc Acad Sci USA* 96:156-160.
- Yamamoto Y, Ohshima K, Kikyo M, Sano T, Nakagami Y, Inoue Y, Nishimura N, Morishima I, Guo D-F, Hamakubo T and Inagami T (1995) Mutagenesis and the molecular modeling of the rat angiotensin II receptor (AT₁). *J Biol Chem* 270:14024-14030.
- Yang CR, Phillips MI and Renaud LP (1992) Angiotensin II receptor activation depolarizes rat sympathetic neurons in vitro. *Am J Physiol Cell Physiol* 263:R1333-R1338.
- Yang H, Lu D, Yu K and Raizada MK (1999) Regulation of the neuromodulatory actions of angiotensin II in brain neurons by the RAS-dependent mitogen-activated protein kinase pathway. *J Neurosci* 19:4047-4055.
- Yee DK, Reagan LP, Mogh CN, Siemieni R and Fluharty SJ (1994) Angiotensin II stabilizes a multimeric type 2 (AT₂) receptor complex in murine neuroblastoma N1E-115 cells. *Regul Pept* 54:655-666.
- Yoshida M, Kikukawa M, Hata H and Satoh S (1996) Modulation of nitric oxide and prostaglandin of the renal vascular response to angiotensin II (3-8). *Br J Pharmacol* 117:885-890.
- Yoshiyama M, Takeuchi K, Omura T, Kim S, Yamagishi H, Tada I, Teragaki M, Aikawa K, Iwano J and Yonikawa J (1989) Effects of nifedipine and clonidine on rats with myocardial infarction assessed by echocardiography. *Hypertension* 38:861-868.
- Yu M, Sandeman S and Unger T (1998) Angiotensin AT₂ receptor mediates expression of Na⁺/HCO₃⁻ cotransporter in rat myocardium after experimental myocardial infarction. *Hypertension* 33:807.
- Yu SS, Lefkowitz RJ and Hausdorff WP (1993) Beta-adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J Biol Chem* 268:337-341.
- Zarbin RD, Ye X, Ades AM, Reagan LP and Fluharty SJ (1992) Angiotensin-induced cyclic GMP production is mediated by multiple receptor subtypes and nitric oxide in N1E-115 Neuroblastoma cells. *J Neurochem* 58:1980-1985.
- Zhang J and Post RH (1990) The AT₂ receptor selectively associates with G_{i2} and G₁₃ in the rat fetus. *J Biol Chem* 265:15028-15033.
- Zhang JR, Hanesworth JM, Sertini M, Ali JA, Wright JW and Harding JW (1999) Structural analysis of angiotensin II receptor (AT₁) from selected bovine tissues. *J Pharmacol Exp Ther* 288:1075-1083.
- Zhou H, Moore GJ and Vogel HU (1991) Protein NMR studies of angiotensin II and its analogs in aqueous solution. *J Protein Chem* 10:383-343.
- Zhou B and Herbert J (1996) Central antagonism of atrial natriuretic peptides on behavioral and hormonal responses to angiotensin II: Mapping with c-Fos. *Brain Res* 734:56-60.
- Zhou B and Herbert J (1997) Angiotensin II interacts with nitric oxide-cyclic GMP pathway in the central control of drinking behaviour: Mapping with c-Fos and NADPH-diaphorase. *Neuroscience* 79:549-558.
- Zhu M, Gelband CH, Posner P and Sumners C (1998a) Angiotensin II type 1 receptor-mediated inhibition of voltage-dependent K⁺ current in cultured neurons: Role of calcium/calmodulin-dependent protein kinase II. *FASEB J* 12:454.
- Zhu M, Neuhoff RB, Wade BM, Posner P, Gelband CH and Sumners C (1997a) Modulation of K⁺ and Ca²⁺ currents in cultured neurons by an angiotensin II type 1 receptor peptide. *Am J Physiol* 273:C1040-C1048.
- Zhu YC, Zhu YC, Gohlo P, Strauss HN and Unger T (1997b) Effects of angiotensin-converting enzyme inhibition and angiotensin II receptor antagonist on cardiac parameters in left ventricular hypertrophy (Review). *Am J Cardiol* 80:116A-117A.
- Zhu YC, Zhang SH, Wagner C, Kartz A, Mowda N, Guffman T and Aronowicz WJ muscle cells of AT₁ receptor-deficient mice. *Hypertension* 31:1171-1177.
- Zhuo J, Alcorn D, Harris PJ and Mendelsohn FAO (1998) Localization and properties of angiotensin II receptors in rat kidney. *Kidney Int* 44 (Suppl 42):140-146.
- Zhuo J, Maric C, Harris PJ, Alcorn D and Mendelsohn FAO (1997) Localization and functional properties of angiotensin II AT₁ receptors in the kidney: Focus on nonmedullary interstitial cells. *Hypertens Res* 20:233-239.
- Zhuo J, Moeller T, Jenkins T, Chai SY, Allen AM, Ohishi M and Mendelsohn FAO (1998) Mapping tissue angiotensin-converting enzyme and angiotensin AT₁, AT₂ and AT₃ receptors. *J Hypertens* 16:2027-2037.
- Zhuo J, Song K, Harris PJ and Mendelsohn FAO (1992) In vitro autoradiography reveals predominantly AT₁ angiotensin II receptor subtypes in rat kidney. *Renal Physiol Biochem* 19:281-289.
- Zini S, Fournie-Zalinski M-C, Chauve P, Roques BP, Corvol P and Lorenz-Cortes C (1996) Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: Predominant role of angiotensin III in the control of vasopressin release. *Proc Natl Acad Sci USA* 93:11988-11993.
- Zini S, Demassay Y, Fournie-Zalinski M-C, Biochiffi I, Corvol P, Lorenz-Cortes C and Sanderson P (1998) Inhibition of vasopressin release by central injection of a specific aminopeptidase A inhibitor. *NeuroReport* 9:525-528.

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT G



The AT2 receptor—A matter of love and hate

U.M. Steckelings¹, E. Kaschina¹, Th. Unger^{*}

Center for Cardiovascular Research, Institut für Pharmakologie und Toxikologie, Charité–Universitätsmedizin Berlin,
Hessische Strasse 3–4, 10115 Berlin, Germany

Available online 18 April 2005

Abstract

In 1989, the development of specific angiotensin receptor antagonists which distinguish between two angiotensin receptor subtypes (AT1 and AT2) led to a breakthrough in angiotensin research. It turned out, that the AT1 receptor was almost entirely responsible for the “classical” actions of angiotensin II related to the regulation of blood pressure as well as volume and electrolyte balance. However, actions and signal transduction mechanisms coupled to the AT2 receptor remained enigmatic for a long time. The present review summarizes the current knowledge of AT2 receptor distribution, signaling and function with an emphasis on growth/anti-growth, differentiation and the regeneration of neuronal tissue.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Angiotensin; AT2 receptor; Renin–angiotensin-system

1. Introduction

There are probably not so many molecules which polarize the “angiotensin-community” as much as the angiotensin AT2 receptor (AT2-R) does. How can this phenomenon be explained? Those scientists, who belong to the group of “AT2 receptor lovers” are often attracted by the many secrets this receptor still holds, and which wait to be disclosed [98]. What could be more tempting and motivating for a scientist than an area of research with many open questions waiting to be dealt with? However, there are reasons why so much is not yet known about this receptor—reasons which prompt some scientists to avoid working on this receptor, if not even “to hate” it.

But what are these reasons? Firstly, the AT2-R is “not easy to find”. In the adult, healthy organism—no matter what species—it is only expressed in certain cell types and certain tissues such as vascular endothelial cells, distinct areas of the brain, the adrenal, selected cutaneous, renal and cardiac

structures, myometrium and ovaries [20,53]. In contrast, in fetal tissues, the AT2-R is the dominating receptor subtype [37]. In the adult, AT2-Rs are re-expressed under pathophysiological conditions such as mechanical injury or ischemia [31,90,72]. For *in vitro* experiments, there is only a limited number of cell types known to express the AT2-R in culture, often only under certain culture conditions. Moreover, primary cell cultures are frequently needed since the number of cell lines expressing the AT2-R is even more limited (for a list of cell-types expressing the AT2-R *in vitro* see Section 2 and de Gasparo et al. [20]).

Secondly, signaling and physiological functions of the AT2-R are unconventional. After it had turned out that the actions of angiotensin II (Ang II) are mediated via at least two distinct receptor subtypes (AT1 and AT2), it was broadly expected that the known actions of Ang II such as blood pressure elevation, sodium and water retention, vasopressin release, cell proliferation or hypertrophy could now be attributed to one or the other receptor. But this was not the case. It turned out that all the established actions of Ang II were mediated by the AT1-R. In terms of AT2-R signaling and function, one had to start from the very beginning.

And, thirdly, AT2-R actions are often “hidden” behind opposing AT1-R actions—meaning that AT2-R functions are often not readily obvious in an experimental setting, but have

^{*} Corresponding author. Tel.: +49 30 450525002; fax: +49 30 450525091.

E-mail addresses: ulrike.steckelings@charite.de (U.M. Steckelings), clem.kaschina@charite.de (E. Kaschina), thomas.unger@charite.de (Th. Unger).

¹ These authors contributed equally to this work.

to be unraveled by blocking the AT1-R. Moreover, AT2-R actions are often inhibitory or counteracting, which requires treatment of the examined organism or cells with the "AT2-R-opposed" stimulus prior to the actual experiment.

No wonder, that after the discovery and characterization of the AT2-R in 1989 [100,14], it took more than five more years until the first data concerning AT2-R signaling and physiological functions were published providing first clues as to what is nowadays widely accepted as AT2-R features [8,71,92]. These features comprise: (a) in terms of signaling that the AT2-R is G-protein coupled but in many cases activates signaling cascades independent of classical regulatory G-proteins, (b) in terms of function the role of a kind of "natural AT1-R antagonist", since in many cases (proliferation/antiproliferation, cell division/differentiation and apoptosis, vasoconstriction/vasodilation) the AT2-R counteracts AT1-R-mediated actions [20].

The present article reviews the current knowledge of AT2-R expression, signaling and function with a focus on the very recently described AT1-R- and AT2-R-binding proteins and on the role of AT2-Rs in neuronal repair and remodeling and in the counterplay of growth and anti-growth processes.

2. Tissue distribution of the AT2 receptor

The AT2 receptor is widely expressed in fetal tissues [37], whereas its expression is dramatically decreased after birth, being restricted to a few organs such as brain, adrenal, heart, vascular endothelium, kidney, myometrium and ovary [20]. The predominant expression of AT2-Rs in differentiated fetal mesenchymes, such as mesenchymal tissues in the tongue, subdermal and subcutaneous regions of the skin and the diaphragm [85], points to the fact that Ang II may use these receptors in development and differentiation processes.

Recent studies mapped the distribution of AT2-Rs in the adult rat brain and found a predominant expression in certain parts of the limbic system, in several thalamic nuclei, the subthalamic nucleus, the inferior olivary complex, the motor hypoglossal nucleus, and numerous brainstem and cerebellar nuclei [58]. This expression-pattern of the AT2-R points to its involvement in a broad spectrum of central actions comprising the sensory and motor systems as well as behavior and emotions.

In the heart, the AT2-R is present in coronary arteries, cardiomyocytes and the ventricular myocardium [99,12].

In the kidney, the expression of AT2-R mRNA is mainly localized in interlobular arteries [66], in the proximal tubules, collecting ducts, arcuate arteries, afferent arterioles and outer medullary descending vasa recta [69]. Possible renal functions of the AT2-R in the regulation of glomerular blood flow and pressure-natriuresis have recently been proposed [13].

In human lung, AT2-R mRNA and protein were demonstrated in the epithelium, with strong staining on the bronchial epithelial cell brush border and also on many of the under-

lying mucous glands. The AT2-R was also present on some endothelial cells [11].

Although the AT1-R is dominant in the adult organism, an increase of AT2-R expression has been observed under pathological conditions, such as vascular injury [71], myocardial infarction [72,108], congestive heart failure [76], renal failure [4], brain ischemia [64], sciatic or optic nerve transection [31,62]. A study from our laboratory employing single cell PCR investigated the expression of AT1-R and AT2-R mRNA in adult cardiomyocytes [12]. The AT2-R gene was transcribed in about 10% of adult rat cardiomyocytes, while about 40% of cardiomyocytes expressed the AT1 receptor. These receptors were not increased on day 1 after tissue injury. However, on day 7 post-infarct, the AT2-R expression was increased to a maximum of 60% AT2-R mRNA-positive cells [63].

For healthy human skin we could show, that the AT2-R is expressed throughout the epidermal layer as well as in certain dermal structures such as vessel walls and sebaceous glands [89]. However, in incisional cutaneous wounds, AT2-R expression is markedly upregulated, an effect which is most pronounced throughout the dermis extending by far the expression-pattern in normal skin [90].

To clarify the signaling mechanism and functional significance of the AT2-R, numerous types of cells derived from tissues known to contain the AT2-R were prepared or identified. Among primary cultured cells were neuronal cells dispersed from rat neonatal hypothalamus [94], neonatal rat cardiomyocytes [65], adult rat cardiac microvascular endothelial cells [92], rat fetal skin cells [96], human adult skin cells [89] and rat fetal fibroblasts [51]. A subline, PC12W, derived from rat pheochromocytoma cells, which expresses a large amount of AT2-Rs but no AT1-Rs [88] was extensively used for studies of AT2 signaling mechanisms and for AT2 cloning. The other cell lines, known to express AT2-Rs, are a subline of the Swiss mouse 3T3 fibroblast cell line, R3T3, [22] and the undifferentiated mouse/rat neuroblastoma × glioma hybrid cell lines, NG108-15 [10] and Neuro-2a [43].

These cell lines have provided useful materials for studies on AT2-R signaling, pathophysiology, regulation and for cloning and purification.

3. AT2-receptor signaling

Knowledge about AT2-R signaling has grown immensely over the last couple of years. Still, it is a very complex issue, since (a) many unconventional signaling cascades seem to be involved, (b) many cascades have not yet been fully defined, and (c) some AT2-R coupled signaling mechanisms could not yet clearly be attributed to certain AT2-R functions and vice versa. But among all this confusion, there is no doubt about one fact: with very few exceptions, signaling via the AT2-R is totally distinct from AT1-R coupled signaling.

In 2000, Nouet and Nahmias suggested that three major transduction mechanisms are responsible for AT2-R

signaling: (a) the activation of various protein phosphatases causing protein dephosphorylation, (b) activation of the NO/cGMP system, and (c) stimulation of phospholipase A2 with subsequent release of arachidonic acid [73]. This classification still seems suitable to range in most current data. However, it has to be supplemented by very recent data claiming that an AT2-R binding protein (termed ATBP or ATIP) functions as an early mediator of growth-inhibitory signaling cascades, probably by modifying AT2-R surface expression [74,103].

3.1. AT2-R signaling via phosphatase activation

Signaling via activation of protein-phosphatases was one of the first AT2-R coupled second messengers suggested, and there is accumulated evidence speaking for a central role of phosphatases for AT2-R signaling, especially for the negative cross-talk of this receptor with growth factors and the AT1-R. In 1992, Bottari et al. reported the involvement of a vanadate-sensitive tyrosine-phosphatase [8]. Up to now, three specific phosphatases could be identified, which are stimulated upon AT2-R activation: mitogen-activated protein kinase phosphatase 1 (MKP-1) [46], SH2 domain-containing phosphatase 1 (SHP-1) [5], and protein phosphatase 2A (PP2A) [47]—and more may follow. MKP-1 and SHP-1 are indeed tyrosine phosphatases and vanadate-sensitive, and Bottari et al. [8] and Brechler et al. [9] probably already saw their effects in the early studies. MKP-1 additionally dephosphorylates threonine-residues, while PP2A is an okadaic acid-sensitive serine/threonine phosphatase [73].

As further elaborated on in Section 4, a major action of AT2-Rs is to counteract growth factor-induced cell proliferation and to induce apoptosis. Growth factors (including Ang II via the AT1-R) mediate their growth promoting actions preferably via tyrosine-kinase receptors and several additional kinase-driven phosphorylation steps further “down” the respective signaling cascades. Extra-cellular signal-regulated kinases 1 and 2 (ERK 1/2) seem to play a key role in these phosphorylation cascades. The AT2-R negatively cross-talks with these phosphorylation cascades by reversion of the phosphorylation steps through phosphatase activation. Most documented is the dephosphorylation and subsequent inhibition of ERK 1/2 as a result of AT2-R activation, and all three AT2-R-activated phosphatases (MKP-1, SHP-1, and PP2A) seem to be involved [46–48;5,27,2]. Since these phosphatases reach their maximal activation at different time points and display divergent dephosphorylation specificities, they may interact with the ERK 1/2 cascade at different steps [73]. But there is also evidence for the dephosphorylation of other targets such as STAT as well as the immediate interaction with receptor tyrosine kinases [45,24].

The inactivation of the MAP-kinases ERK 1/2 by dephosphorylation seems to be a key messenger not only in AT2-R-mediated growth inhibition, but also in its proapoptotic effect, which could be shown to depend on activation of MKP-1

and SHP-1 and, subsequently, on Bcl-2 dephosphorylation by MKP-1 and upregulation of bax [44].

Interestingly, the AT2 receptor seems to modulate ERK activity depending on the overall cellular input: while an NGF-mediated ERK stimulation was inhibited by costimulation with Ang II in PC12W cells, stimulation with Ang II alone initially enhanced ERK activity in these cells under conditions which enable neurite outgrowth [93].

3.2. G-protein coupling of AT2-receptors

Although cloning and sequencing of the AT2-R revealed a hepta-helical molecule comprising 363 amino acids which is structurally related to G-protein coupled receptors, the signaling mechanisms of the receptor seem to involve unconventional second messengers in addition to more classical G-protein coupled pathways [20].

In some cases, AT2-Rs are coupled to G_{i2-} and G_{i3-} proteins [52,106]. However, activation of the tyrosine phosphatase SHP-1 seems to depend on the $G_{\beta\gamma}$ -independent constitutive association of $G_{\alpha s}$ with SHP-1 and the AT2-R. In a non-activated state, SHP-1 is kept inactive through interaction of two SH2 domains, which are part of the SHP-1 molecule itself, with its catalytic domain. Upon ligand binding to the AT2-R, SHP-1 dissociates from the receptor and is activated by removal of the self-constrain imposed by the SH2 domains [25].

3.3. AT2-R signaling via activation of the NO/cGMP system

Activation of NO release with a subsequent increase in intracellular cGMP levels is one of very few – if not the only—signaling pathway(s), which are/is shared by AT1- and AT2-Rs [42]. An AT2-R coupled increase in NO generation could be observed in vascular tissue of diverse origin such as cultured bovine aortic endothelial cells [102,81], dog coronary microvessels or large coronary arteries [84], isolated perfused rat renal arteries [84,95], but also in the neuronal cell lines PC12W [107] and NG-108-15 [15,35], in which this signaling cascade promotes differentiation and neuronal outgrowth. In the vasculature, this signaling cascade may mediate vasorelaxation induced via AT2-Rs (for review see [101]), a common result of NO production, while in the kidney it has been attributed to natriuresis [87].

In some tissues, the AT2-R induced increase in NO synthesis seems to depend on the release of endogenous bradykinin. In stroke-prone spontaneously hypertensive rats (but not in normotensive Wistar Kyoto rats), acute Ang II infusion produced a significant increase in aortic cyclic GMP via a mechanism that involved AT2-Rs, endothelium-derived NO and the bradykinin B2 receptor [36,77]. Accordingly, in conscious rats, endogenous Ang II as well as infused Ang II caused an increase in cyclic GMP content in interstitial renal fluid, an effect that could be abolished by AT2-R blockade as well as bradykinin B2-receptor blockade [86]. Looking on genetic

models, AT2-R-null mice exhibit markedly reduced basal- and Ang II-induced cyclic GMP and bradykinin levels in renal interstitial fluid [86], and in arteries isolated from kininogen-deficient Brown Norway Katholic rats, the AT2-R-mediated vasodilator response was markedly impaired compared to their wild type counterparts [54], again suggesting an important role for bradykinin in the signaling cascade leading from AT2-R stimulation to the vasodilatory response.

Despite a growing body of data on this topic, the AT2-R/NO/cGMP cascade is not yet fully understood. Especially the way, in which AT2-R stimulation may activate bradykinin release is still a matter of speculation, although there are data suggesting that Ang II via the AT2-R causes a reduction of intracellular pH levels (a mechanism, which may also be important for the regulation of intracellular acidosis following injury [82]), which in turn leads to the activation of acid-optimizing kininogenases to cleave bradykinin from kininogen [102,97].

However, the AT2-R-coupled increase in cGMP levels does not necessarily require bradykinin since in FC12W cells, cGMP levels were enhanced upon AT2-R stimulation in the presence of B2-receptor blockade [107], and these data were confirmed by Hannan et al. [41] in rat uterine arteries.

3.4. Angiotensin receptor binding proteins

Recently, Guo et al. identified and sequenced a novel AT1-R binding protein (ARAP1), which does not belong to the G-protein family and which promotes recycling of the AT1a-R to the plasma membrane [39].

A similar protein seems to exist (ATBP50), which is Golgi-membrane-associated, but on activation binds to the cytoplasmic carboxy-terminal AT2-R and regulates the transport of the AT2-R to the cell membrane, thus modulating AT2-R expression at the cell surface [103]. The level of receptor expression seems to be critical for the induction of anti-proliferation and apoptosis, since the inhibitory effect mediated by AT2-Rs at least in part depends on the presence and density, but not on the extracellular activation of the receptor [68].

Nouet et al. cloned an identical protein, termed it ATIP1, and showed that in the presence of AT2-Rs, this protein inhibits insulin-, basic fibroblast growth factor-, and epidermal growth factor-induced ERK1/2 activation and cell proliferation, and attenuates insulin receptor autophosphorylation. Binding of Ang II to the AT2-R reinforces this effect, but ligand binding is not necessarily required. It is not clear yet, which intracellular steps are intercalated between ATIP/ATBP50 activation and ERK 1/2 inhibition. Nouet et al. could rule out the involvement of the phosphatase SHP-1 and of a direct interaction with growth factor receptors. However, other phosphatases or still unknown signaling mechanisms could play a role. But there is also the possibility that ATIP/ATBP50 simply acts by "intracellular activation" of AT2-Rs.

In contrast to the AT2-R, ATIP1/ATBP50 is ubiquitously distributed in the adult organism. A fact that may point to fur-

ther roles of this protein inside the cell, which extend AT2-R expression and signaling, but it can also be speculated, that ATIP1/ATBP50 is required and responsible under pathophysiological conditions such as injury or infarction to re-express AT2-Rs, a phenomenon, which can be observed in almost any tissue.

3.5. AT2-R signaling via activation of phospholipase A2

In vivo studies have shown that Ang II via the AT2 receptor activates phospholipase A2 [40]. The resulting release of arachidonic acid may contribute to the activation of a $\text{Na}^+/\text{HCO}_3^-$ symporter system (NBC), which regulates intracellular pH [36,82]. In renal cells, arachidonic acid released upon AT2-R stimulation and cytochrome P450-dependent metabolites generated from arachidonic acid have been shown to activate MAP-kinases and additional kinases further downstream the RAS-signaling pathway—one of the few cases in which the AT2-R is coupled to kinase- and not phosphatase-activation [50,23,93].

3.6. Further pieces of the puzzle

3.6.1. Ceramides and caspases

Ceramides are intracellular lipid second messengers, which were originally characterized as being part of cytokine signaling. Recently, our group has demonstrated, that ceramides are de novo synthesized upon AT2-R activation, probably mediating a pro-apoptotic stimulus via activation of caspase 3 [32]. Caspase 3 had previously been shown, too, to be upregulated via AT2-Rs and to mediate apoptosis [21]. Lehtonen et al. described the same pro-apoptotic pathway and provided further evidence for the assumption that AT2-R-induced apoptosis depends on the de-novo synthesis of ceramides by showing that blockade of sphingolipid synthesis abolished AT2-R-mediated programmed cell death [59].

3.6.2. Zinc-finger

Recently, we identified a zinc-finger homeodomain enhancer binding protein gene (*Zfhep*) that is induced after activation of the AT2-R in cells of neuronal and vascular origin [91]. This gene encodes for a transcription factor recognized for its role in the regulation of cellular differentiation, and is expressed under experimental conditions where the AT2-R exerts its antiproliferative and differentiation-promoting effects. *Zfhep* not only represents a suitable marker for AT2-R activation but may also link AT2-R signaling and downstream events involved in the proposed function of the AT2-R in development and regeneration.

4. Functional properties of the AT2 receptor

Significant progress has been made lately in the elucidation of the functional properties of the AT2-R.

Physiologically, the AT₂-R appears to act as a modulator of complex biological programs involved in embryonic development, cell differentiation, apoptosis, regulation of renal function and blood pressure [20,98,13]. Moreover, the AT₂-R is involved in different pathophysiological processes such as cardiovascular remodeling following myocardial infarction and hypertension, heart failure and stroke, and it can be speculated that AT₂-R-mediated effects are even more apparent in pathological situations in which these receptors are upregulated. The current knowledge including yet conflicting data has been discussed in detail in recent reviews [55,101].

It seems that one major task of AT₂-Rs consists in a protective effect against an overstimulation of AT₁-Rs, and these opposing effects may occur beyond the level of the cell membrane receptor as has been commented on in Section 3. For example, regression of cardiac fibrosis and vascular remodeling evoked by AT₂-Rs oppose the profibrotic AT₁-R-mediated action of Ang II [104,105]. It has also been reported that vasodilatory effects of Ang II linked to the AT₂-R oppose the vasoconstrictor actions of Ang II-mediated by the AT₁-R [13]. Furthermore, AT₂-Rs may play a role in pressure natriuresis, opposing the antinatriuretic effects of AT₁-R activation [87].

The contribution of the AT₂-R to the regeneration processes in the nervous system still represents a new aspect of investigations, which is closely connected with the role of the AT₂-R in cell growth and differentiation. Recent studies in the following two directions will be discussed in more detail in the following.

4.1. Growth and anti-growth

An antiproliferative effect of the AT₂-R in cultured coronary endothelial cells was first observed by Stoll et al. [92]. We and others were able to extend these results to macrovascular endothelial cells from bovine aorta, microvascular endothelial cells from human dermis as well as to cells of neuronal origin [91,57,71,263,67]. In further *in vivo* studies, Munzmaier and Greene [70] reported opposing effects of AT₁- and AT₂-Rs on microvascular growth in the rat cremaster muscle. In their study, Ang II induced angiogenesis in the microvasculature through the AT₁-R, and this effect was buffered by the AT₂-R. Recent studies extended these data in terms of underlying mechanisms. A study from our laboratory suggested that upregulation of thrombospondin-1 expression may be involved in the antiproliferative effect of the AT₂-R on bovine, macrovascular endothelial cells [26]. In cardiac microvascular endothelial cells, AT₁- and AT₂-Rs differentially regulated angiotensin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding epidermal growth factor (EGF)-mediated EGF receptor transactivation [29]. Thus, from the clinical point of view, AT₁-R antagonists may act inhibitory on neovascularization processes by unmasking the antiproliferative effect of AT₂-Rs. This has been observed recently in the model of hyperoxia- and normoxia-induced retinal neovas-

cularization in newborn mice [61]. There are, however, conflicting data, claiming that AT₂-R blockade reduces retinal angiogenesis and alters the expression of vascular endothelial growth factor and angiotensin-Tie2 [83]. Still, inhibition of neovascularization may be beneficial in the prevention of proliferative retinopathies such as proliferative diabetic retinopathy. Moreover, it has been reported that the AT₁-R antagonist TCV-116 inhibited tumor-associated angiogenesis, tumor growth, and metastasis in a murine model [28]—a fact that may achieve even more clinical relevance.

Myointimal hyperplasia occurs under pathophysiological conditions such as endothelial injury. A contribution of Ang II to the process of neointima formation after vascular injury has been suggested in studies showing that ACE inhibitors can attenuate the development of myointimal hyperplasia after endothelial denudation [78]. Nakajima et al. [71] transfected an AT₂-R expression vector into a balloon-injured rat carotid artery *in vivo* and found that overexpression of the AT₂-R attenuated neointima formation. Recent studies confirmed these data. Akishita et al. [1] demonstrated that the AT₂-R antagonist PD 123319 increased neointima formation in the cuff-induced vascular injury model in mice. Furthermore, treatment with even small doses of the AT₁-R antagonist valsartan effectively attenuated neointima formation, whereas this effect was significantly weaker in AT₂-null mice [105]. Thus, the beneficial effect of AT₁ blockade in the prevention of neointima formation seems to involve AT₂-R stimulation.

4.2. Role in the regeneration of the nervous system

Over the last decade, the AT₂-R has been shown to be involved in various processes in the nervous system (for review see [17,80,34]).

As stated more detailed above, in contrast to AT₁-Rs, which are present in the majority of brain areas, AT₂-Rs are limited to distinct regions, and their expression is low in peripheral nerves [75,31]. However, under pathological conditions such as stroke [108,64], sciatic [31] or optic [62] nerve transection, and Huntington's or Alzheimer's disease [33], an increased AT₂-R expression has been described, pointing to a possible involvement of this receptor in maintaining functions of the central nervous system (CNS).

In vitro evidence for a role of AT₂-Rs in the repair and remodelling of neuronal tissue originates from experiments showing that Ang II via its AT₂-R can induce neuronal differentiation in PC12W cells [67,30,93] and neurite outgrowth in the neuroblastoma × glioma hybrid cell line NG 108-15 [57]. The presence of both AT₁- and AT₂-Rs was demonstrated in cultured Schwann cells as well as in the rat sciatic nerve [6]. A further step was the observation that axotomy and nerve crush resulted in a time-dependent upregulation of AT₂-R mRNA in sciatic nerve segments coinciding with the successful regeneration of nerve fibers [31]. Moreover, AT₂-R expression was observed not only at the site of injury, but also in the appropriate dorsal root ganglia neurons [31].

Recently, we could provide direct evidence that Ang II via its AT₂-R not only promotes regeneration but also functional recovery after sciatic nerve crush in rats [79]. Ang II applied locally accelerated functional recovery which was evaluated by the re-innervation parameters toe spread distance and response to local electrical stimulation. The AT₂-R antagonist PD 123319 administered alone or in combination with Ang II completely prevented the Ang II-induced recovery, whereas the AT₁-R antagonist losartan had no effect. Ang II also induced AT₂-R-mediated activation of the transcription factor NF- κ B in Schwann cells leading to enhanced remyelination.

We have demonstrated that stimulation of AT₂-Rs on retinal ganglion cells induced a significant neurite outgrowth in retinal explant cultures *in vitro* and after a lesion of the optic nerve also *in vivo* [62].

To investigate the mechanisms underlying Ang II-induced neurite outgrowth and elongation, several studies have been performed in rat neuronal cells in culture. The stability and function of neurons are dependent on protein filaments like microtubules, actin- and intermediate filaments. Our group examined the effect of AT₂-R stimulation on the microtubule components, NF-1, beta-tubulin, MAP1B and MAP2, in quiescent and nerve growth factor (NGF)-differentiated PC12W cells. The results implicated a role of AT₂-Rs in cell differentiation and nerve regeneration via regulation of the cytoskeleton [93]. Cote et al. [16] studied the morphological differentiation of cerebellar cells. A selective activation of the AT₂-R induced an increased elongation of neurites, cell migration and an increase in the expression of neuron-specific beta III-tubulin and the microtubule-associated proteins tau and MAP2.

Possible signaling pathways associated with AT₂-R-mediated neuroregeneration include the bradykinin/NO/cGMP signaling cascade [107], expression of *Zfp*, a zinc-finger homeodomain enhancer-binding protein [91], Rap1/B-Raf, and MAPK (also see Section 3 and for review [34,48]).

Apparently, both Ang II receptors take part in the pathophysiological events initiated by central ischemia and stroke. Experimental evidence comes from studies showing that chronic central pretreatment with the AT₁-R antagonists irbesartan and losartan improved the neurological outcome and reduced cortical and hippocampal AP-1 transcription factor expression after focal brain ischemia in the rat [19], and that AT₂-Rs contributed to the observed beneficial effects of AT₁-R antagonist [7].

Systemic pretreatment with the AT₁-R antagonist candesartan also improved recovery from brain ischemia [38]. These data are consistent with recent results from clinical trials demonstrating a reduction of stroke incidence in patients receiving the AT₁ antagonist losartan [18].

Recent evidence points to an upregulation of neuronal AT₂-Rs in response to cerebral ischemia in rats in some brain regions. The enhanced AT₂-R expression was associated with intense neurite outgrowth [60]. Furthermore, by central co-administration of AT₁- and AT₂-R-antagonists, we

could show that cerebral intact, i.e. unopposed AT₂-Rs were a necessary requirement for the neuroprotective effects of central AT₁-R blockade such as reduction of infarct size and improved neurological outcome [60]. These data are consistent with the observations by Iwai et al., who compared infarct size and neurological outcome after middle cerebral artery occlusion in Agtr2(-/-) versus Agtr2(+/-) mice [49]. Thus, cerebral AT₂-Rs seem to exert a neuroprotective effect.

Taken together, although the cellular targets of the AT₂-R in neuronal tissue are not yet clearly identified, the findings on the neurotrophic actions of AT₂-R stimulation may provide a basis for the design of new therapeutic concepts for treatment of human peripheral nerve injuries, diabetic neuropathy, neurodegenerative disorders and stroke.

5. Conclusions

Fifteen years and more than 2000 respective publications have gone by since the discovery of the AT₂-R. Still, we are far from really knowing this receptor. May be, one main reason for this is the fact, that we are used to deal with "active" receptors, receptors which positively evoke actions, and not with a mainly inhibitory or modulating receptor as the AT₂-R in most cases seems to be. But modulation is an important capability of organisms or cells for the "fine-tuning" of a broad spectrum of actions, which – in case of the AT₂-R – seems to span the regulation of natriuresis, body-temperature, blood-pressure, reproduction, embryonic development, cell differentiation, tissue repair and programmed cell death.

To better understand the AT₂-R as a modulator of complex biological programs as such is a rewarding objective. But since drugs interfering with the renin-angiotensin-system belong to the most commonly used cardiovascular medications and thus to the most frequently prescribed drugs, the knowledge of AT₂-R actions has also important clinical implications, because these actions are concomitantly either blocked (ACE-inhibitors) or stimulated (AT₁-R-antagonists) together with the actual goal of the therapy, which is the inhibition of AT₁-R-mediated actions.

References

- [1] Akishita M, Horiuchi M, Yamada H, Zhang L, Shirakami G, Tamura K, et al. Inflammation influences vascular remodeling through AT₂ receptor expression and signaling. *Physiol Genomics* 2002;13:20.
- [2] Akishita M, Ito M, Lehtonen JY, Daviet L, Dzau VJ, Horiuchi M. Expression of the AT₂ receptor developmentally programs extracellular signal-regulated kinase activity and influences fetal vascular growth. *J Clin Invest* 1999;103:63–71.
- [3] Artuc M, Henz BM, Stoll M, Grützkau A, Steckelings UM. Role of angiotensin AT₁- and AT₂ receptors on proliferation of human dermal microvascular endothelial cells. *International Workshop on endothelial Cells*, Innsbruck, Oktober. *Exp Dermatol* 1997;6:279.
- [4] Bautista R, Sanchez A, Hernandez J, Oyskan A, Escalante B. Angiotensin II type AT₂ receptor mRNA expression and

- renal vasodilatation: are increased in renal failure. *Hypertension* 2001;38:669–73.
- [5] Bedecs K, Elbaz N, Sutren M, Masson M, Suxini C, Siroberg AD, et al. Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. *Biochem J* 1997;325(Pt 2):449–54.
 - [6] Blouet A, de Gasparo M, Whitebread S, Puttner I, Monard D. Regulation of protease nexin-1 expression in cultured Schwann cells is mediated by angiotensin II receptors. *J Neurosci* 1995;15:750–61.
 - [7] Blume A, Funk A, Gohlke P, Unger JH, Culman J. AT2 Receptor inhibition in the rat brain reverses the beneficial effects of AT1 receptor blockade on neurological outcome after focal brain ischemia. *Hypertension* 2000;36:356 (abstract).
 - [8] Bottari SP, Obermüller N, Bogdal Y, Zahs KR, Descheppe CF. Characterization and distribution of angiotensin II binding sites in fetal and neonatal astrocytes from different rat brain regions. *Brain Res* 1992;585:372–6.
 - [9] Brechler Y, Reichlin S, De Gasparo M, Bottari SP. Angiotensin II stimulates protein tyrosine phosphatase activity through a G-protein independent mechanism. *Recept Channels* 1994;2:89–98.
 - [10] Buisson B, Bottari SP, de Gasparo M, Gallo-Payet N, Payet MD. The angiotensin AT2 receptor modulates T-type calcium current in non-differentiated NG108-15 cells. *FEBS Lett* 1992;309:161–4.
 - [11] Bullock GR, Steyer JA, Bilbe G, Carey RM, Kips J, De Paep B, et al. Distribution of type-1 and type-2 angiotensin receptors in the normal human lung and in lungs from patients with chronic obstructive pulmonary disease. *Histochem Cell Biol* 2001;115:117–24.
 - [12] Busche S, Gallinat S, Bohle RM, Reinecke A, Seebeck J, Franke F, et al. Expression of angiotensin AT(1) and AT(2) receptors in adult rat cardiomyocytes after myocardial infarction. A single-cell reverse transcriptase-polymerase chain reaction study. *Am J Pathol* 2000;157:605–11.
 - [13] Carey RM, Wang ZQ, Singay HM. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension* 2000;35:55–63.
 - [14] Chiu AT, Harbin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, et al. Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun* 1989;165:196–203.
 - [15] Cote F, Lafamme L, Payet MD, Gallo-Payet N. Nitric oxide, a new second messenger involved in the action of angiotensin II on neuronal differentiation of NG108-15 cells. *Endocr Res* 1998;24:403–7.
 - [16] Cote F, Do TH, Lafamme L, Gallo JM, Gallo-Payet N. Activation of the AT(2) receptor of angiotensin II induces neurite outgrowth and cell migration in microexplant cultures of the cerebellum. *J Biol Chem* 1999;274:31586–92.
 - [17] Culman J, Baumann J, Blume A, Unger T. The renin-angiotensin system in the brain: an update. *JRAAS* 2001;2:96–102.
 - [18] Dahlöf B, Devereux RB, Kjeldsen SE, Julius S, Boerwag G, Faire U, et al. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet* 2002;359:995–1003.
 - [19] Dai WJ, Funk A, Herdeggen T, Unger T, Culman J. Blockade of central angiotensin AT(1) receptors improves neurological outcome and reduces expression of AP-1 transcription factors after focal brain ischemia in rats. *Stroke* 1999;30:2391–8.
 - [20] de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 2000;52:415–72.
 - [21] Dimmeler S, Rippmann V, Weiland U, Haendeler J, Zeiher AM. Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide. *Circ Res* 1997;81:970–6.
 - [22] Dudley DT, Hubbell SE, Surmeier RM. Characterization of angiotensin II (AT2) binding sites in R373 cells. *Mol Pharmacol* 1991;40:360–7.
 - [23] Dulin NG, Alexander LD, Harwalter S, Falck JR, Douglas JA. Phospholipase A2-mediated activation of mitogen-activated protein kinase by angiotensin II. *Proc Natl Acad Sci USA* 1998;95:8098–102.
 - [24] Elbaz N, Bedecs K, Masson M, Sutren M, Siroberg AD, Nalmias C. Functional trans-inactivation of insulin receptor kinase by growth-inhibitory angiotensin II AT2 receptor. *Mol Endocrinol* 2000;14:795–804.
 - [25] Feng YH, Sun Y, Douglas JG. Gbeta gamma -independent constitutive association of Galpha s with SHP-1 and angiotensin II receptor AT2 is essential in AT2-mediated ITIM-independent activation of SHP-1. *Proc Natl Acad Sci USA* 2002;99:12049–54.
 - [26] Fischer JW, Stoll M, Hahn AW, Unger T. Differential regulation of thrombospondin-1 and fibronectin by angiotensin II receptor subtypes in cultured endothelial cells. *Cardiovasc Res* 2001;51:784–91.
 - [27] Fischer TA, Singh K, O'Hara DS, Koye DM, Kelly RA. Role of AT1 and AT2 receptors in regulation of MAPKs and MKP-1 by ANG II in adult cardiac myocytes. *Am J Physiol* 1998;275:H406–16.
 - [28] Fujita M, Hayashi I, Yamashita S, Itoan M, Majima M. Blockade of angiotensin AT1a receptor signaling reduces tumor growth, angiogenesis, and metastasis. *Biochem Biophys Res Commun* 2002;294:441–7.
 - [29] Fujiyama S, Matsubara H, Nozawa Y, Maruyama K, Mori Y, Tsutsumi Y, et al. Angiotensin AT(1) and AT(2) receptors differentially regulate angiotensin II and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF)-mediated EGF receptor transactivation. *Circ Res* 2001;88:22–9.
 - [30] Gallinat S, Csikos T, Meffert S, Herdeggen T, Stoll M, Unger T. The angiotensin AT2 receptor down-regulates neurofilament M in PC12W cells. *Neurosci Lett* 1997;227:29–32.
 - [31] Gallinat S, Yu M, Dorst A, Unger T, Herdeggen T. Sclerotic nerve transection evokes lasting up-regulation of angiotensin AT2 and AT1 receptor mRNA in adult rat dorsal root ganglia and sciatic nerves. *Brain Res Mol Brain Res* 1998;57:111–22.
 - [32] Gallinat S, Busche S, Schütz S, Krönke M, Unger T. AT2 receptor stimulation induces generation of ceramides in PC12W cells. *FEBS Lett* 1999;443:75–9.
 - [33] Ge J, Barnes NM. Alterations in angiotensin AT1 and AT2 receptor subtype levels in brain regions from patients with neurodegenerative disorders. *Eur J Pharmacol* 1996;297:299–306.
 - [34] Gendron L, Payet MD, Gallo-Payet N. The angiotensin type 2 receptor of angiotensin II and neuronal differentiation: from observations to mechanisms. *J Mol Endocrinol* 2003;31:359–72.
 - [35] Gendron L, O'Hilly JR, Payet MD, Gallo-Payet N. Cyclic AMP-independent involvement of Rap1/β-Raf in the angiotensin II AT2 receptor signaling pathway in NG108-15 cells. *J Biol Chem* 2003;278:3606–14.
 - [36] Gohlke P, Poes C, Unger T. AT2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. *Hypertension* 1998;31:349–55.
 - [37] Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE. Expression of AT2 receptors in the developing rat fetus. *J Clin Invest* 1991;88:921–33.
 - [38] Groth W, Blume A, Gohlke P, Unger T, Culman J. Chronic pre-treatment with candesartan improves recovery from focal cerebral ischemia in rats. *J Hypertens* 2003;21:2175–82.
 - [39] Guo DF, Chenier I, Tardif V, Orlov SN, Inagami T. Type 1 angiotensin II receptor-associated protein ARAP1 binds and recycles the receptor to the plasma membrane. *Biochem Biophys Res Commun* 2003;310:1254–65.
 - [40] Halilovic D, Jiao H, Cui XL, Hoger U, Douglas JG. Renal proximal tubule AT2 receptor: signaling and transport. *J Am Soc Nephrol* 1999;10(Suppl. 11):S69–74.
 - [41] Haun RR, Davis EA, Widig RR. Functional role of angiotensin II AT2 receptor in modulation of AT1 receptor-mediated contraction

- in rat uterine artery: involvement of bradykinin and nitric oxide. *Br J Pharmacol* 2003;140:987–95.
- [42] Hannan RB, Gaspari TA, Davis BA, Widdop RE. Differential regulation by AT(1) and AT(2) receptors of angiotensin II-stimulated cyclic GMP production in rat uterine artery and aorta. *Br J Pharmacol* 2004;141:1024–31.
- [43] Hoffmann A, Cool DR. Angiotensin II receptor types 1A, 1B, and 2 in murine neuroblastoma Neuro-2a cells. *J Recept Signal Transduct Res* 2003;23:11–21.
- [44] Horiuchi M, Akishima M, Dzau VI. Molecular and cellular mechanism of angiotensin II-mediated apoptosis. *Endocr Res* 1998;24:307–14.
- [45] Horiuchi M, Hayashida W, Akishima M, Tamura K, Daviet L, Lehtonen JY, et al. Stimulation of different subtypes of angiotensin II receptors, AT1 and AT2 receptors, regulates STAT activation by negative cross-talk. *Circ Res* 1999;84:876–82.
- [46] Horiuchi M, Hayashida W, Kambe T, Yamada T, Dzau VI. Angiotensin type 2 receptor dephosphorylates Bad-1 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis. *J Biol Chem* 1997;272:19022–6.
- [47] Huang XC, Richards EM, Summers C. Angiotensin II type 2 receptor-mediated stimulation of protein phosphatase 2A in rat hypothalamic/brainstem neuronal cocultures. *J Neurochem* 1995;65:2131–7.
- [48] Huang XC, Richards EM, Summers C. Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. *J Biol Chem* 1996;271:15635–41.
- [49] Iwai M, Liu HW, Chen R, Ide A, Okamoto S, Hata R, et al. Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation. *Circulation* 2004;110:843–8.
- [50] Jiao H, Cai XL, Torti M, Chang CH, Alexander LD, Lapetina EG, et al. Arachidonic acid mediates angiotensin II effects on p21ras in renal proximal tubular cells via the tyrosine kinase-Shc-Grb2-Sos pathway. *Proc Natl Acad Sci USA* 1998;95:7417–21.
- [51] Johnson MC, Aguilera G. Angiotensin-II receptor subtypes and coupling to signaling systems in cultured fetal fibroblasts. *Endocrinology* 1991;129:1266–74.
- [52] Kang J, Pomer P, Summers C. Angiotensin II type 2 receptor stimulation of neuronal K⁺ currents involves an inhibitory GTP binding protein. *Am J Physiol* 1994;267:1389–97.
- [53] Kaschiba H, Unger T. Angiotensin AT1/AT2 receptors: regulation, signaling and function. *Blood Press* 2003;12:70–88.
- [54] Katada J, Majima M. AT(2) receptor-dependent vasodilation is mediated by activation of vascular kinase generation under flow conditions. *Br J Pharmacol* 2002;136:484–91.
- [55] Kim S, Jwong H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000;52:11–34.
- [56] Kohout TA, Rogers TB. Angiotensin II activates the Na⁺/HCO₃⁻ symport through a phosphoinositide-dependent mechanism in cardiac cells. *J Biol Chem* 1995;270:20432–8.
- [57] Lafamme L, Gasparo M, Gallo JM, Payet MD, Gallo-Payet N. Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells. Effect counteracted by the AT1 receptors. *J Biol Chem* 1996;271:22729–35.
- [58] Lenkei Z, Palkovits M, Corvol P, Llorens-Cortes C. Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review. *Front Neuroendocrinol* 1997;18:383–439.
- [59] Lehtonen JY, Horiuchi M, Daviet L, Akishima M, Dzau VI. Activation of the de novo biosynthesis of sphingolipids mediates angiotensin II type 2 receptor-induced apoptosis. *J Biol Chem* 1999;274:16901–6.
- [60] Li J, Culman J, Hörtnagl H, Zhao Y, Nadezhda G, Timm M, et al. Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury. *FASEB J* 2005;19:617–9.
- [61] Lonchamp M, Pennel L, Duhaoui J. Hyperoxia/aerocytosis-driven retinal angiogenesis in mice: a role for angiotensin II. *Invest Ophthalmol Vis Sci* 2001;42:429–32.
- [62] Lucius R, Gallinat S, Rosenstiel P, Herdogen T, Sievers J, Unger T. The angiotensin II type 2 (AT2) receptor promotes axonal regeneration in the optic nerve of adult rats. *J Exp Med* 1998;188:661–70.
- [63] Luther C, Bohle R-M, Fink L, Zhao Y, Sindermann S, Busche S, et al. Expression of angiotensin AT1 and AT2 receptors in heart cells after myocardial infarction. *Hypertension* 2000;36:655. Abstract.
- [64] Malino I, Shibata K, Ohgami Y, Fujiwara M, Furukawa T. Transient upregulation of the AT2 receptor mRNA level after global ischemia in the rat brain. *NeuroPeptides* 1996;30:596–601.
- [65] Matsubara H, Kanesaki M, Murasawa S, Tsukaguchi Y, Nio Y, Inada M. Differential gene expression and regulation of angiotensin II receptor subtypes in rat cardiac fibroblasts and cardiomyocytes in culture. *J Clin Invest* 1994;93:1592–601.
- [66] Matsubara H, Sugaya T, Murasawa S, Nozawa Y, Mori Y, Masaki H, et al. Tissue-specific expression of human angiotensin II AT1 and AT2 receptors and cellular localization of subtype mRNAs in adult human renal cortex using *in situ* hybridization. *Nephron* 1998;80:25–34.
- [67] Meffert S, Stoll M, Steckelings UM, Bottari SP, Unger T. The angiotensin II AT2 receptor inhibits proliferation and promotes differentiation in PC12W cells. *Mol Cell Endocrinol* 1996;122:59–67.
- [68] Miura S, Kanik SS. Ligand-induced signals from angiotensin II type 2 receptor induce apoptosis. *Embo J* 2000;19:4026–35.
- [69] Miyata N, Park F, Li XF, Cowley Jr AW. Distribution of angiotensin AT1 and AT2 receptor subtypes in the rat kidney. *Am J Physiol* 1999;277:F437–46.
- [70] Munzemaier DH, Greene AS. Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension* 1996;27:760–5.
- [71] Nakajima M, Hutchinson HG, Fujinaga M, Hayashida W, Morishita R, Zhang L, et al. The angiotensin II type 2 (AT2) receptor antagonizes the growth effects of the AT1 receptor: gain-of-function study using gene transfer. *Proc Natl Acad Sci USA* 1995;92:10663–7.
- [72] Nio Y, Matsubara H, Murasawa S, Kanesaki M, Inada M. Regulation of gene transcription of angiotensin II receptor subtypes in myocardial infarction. *J Clin Invest* 1995;95:546–54.
- [73] Nouet S, Nakamas C. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab* 2000;11:1–6.
- [74] Nouet S, Amzallag N, Li J-M, Louis S, Seitz I, Cui T-X, et al. Trans-inactivation of receptor tyrosine kinases by novel angiotensin II AT2 receptor-interacting protein, ATIP. *J Biol Chem* 2004;279:28989–97.
- [75] Obermüller N, Unger T, Culman J, Gohlke P, de Gasparo M, Bottari SP. Distribution of angiotensin II receptor subtypes in rat brain nuclei. *Neurosci Lett* 1991;132:11–5.
- [76] Okubo N, Matsubara H, Nozawa Y, Mori Y, Murasawa S, Kijima K, et al. Angiotensin type 2 receptors are reexpressed by cardiac fibroblasts from failing myopathic hamster hearts and inhibit cell growth and fibrillar collagen metabolism. *Circulation* 1997;96:394–402.
- [77] Pesi C, Unger T, Gohlke P. Effect of angiotensin AT2 receptor stimulation on vascular cyclic GMP production in normotensive Wistar Kyoto rats. *Int J Biochem Cell Biol* 2003;35:963–72.
- [78] Powell JS, Clozel JE, Müller RK, Kuhn H, Hsü F, Hossang M, et al. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* 1989;245:186–8.
- [79] Reinecke K, Lucius R, Reinecke A, Rieckert U, Herdogen T, Unger T. Angiotensin II accelerates functional recovery in the sciatic nerve *in vivo*: role of the AT2 receptor and the transcription factor NF-κB. *FASEB J* 2003;17:2094–6.
- [80] Rosenstiel P, Gallinat S, Arlt A, Unger T, Sievers J, Lucius R. Angiotensin AT2 receptor ligands: do they have potential as future treatments for neurological diseases? *CNS Drugs* 2002;16:145–53.

- [81] Saito S, Hirata Y, Emori T, Imai T, Marumo F. Angiotensin II activates endothelial constitutive nitric oxide synthase via AT1 receptors. *Hypertens Res* 1996;19:201–6.
- [82] Sandmann S, Yu M, Kaschina E, Blume A, Bouzina E, Aalkjaer C, et al. Differential effects of angiotensin AT1 and AT2 receptors on the expression, translation and function of the Na⁺–H⁺ exchanger and Na⁺–HCO₃⁻ symporter in the rat heart after myocardial infarction. *J Am Coll Cardiol* 2001;37:2154–65.
- [83] Sarlos S, Rizkalla B, Moneski C, Cao Z, Cooper ME, Wilkinson-Berka JL. Retinal angiogenesis is mediated by an interaction between the angiotensin type 2 receptor, VEGF, and angiotensin. *Am J Pathol* 2003;163:879–87.
- [84] Seyedi N, Xu X, Nasjletti A, Hintze TH. Coronary kinin generation mediates nitric oxide release after angiotensin receptor stimulation. *Hypertension* 1995;26:164–70.
- [85] Shanmugam S, Lenkel ZG, Gasc JM, Corvol PL, Llorens-Cortes CM. Ontogeny of angiotensin II type 2 (AT2) receptor mRNA in the rat. *Kidney Int* 1995;47:1095–100.
- [86] Siragy HM, Carey RM. The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest* 1997;100:264–9.
- [87] Siragy HM, Carey RM. Protective role of the angiotensin AT2 receptor in a renal wrap hypertension model. *Hypertension* 1999;33:1237–42.
- [88] Speth RC, Kim KH. Discrimination of two angiotensin II receptor subtypes with a selective agonist analogue of angiotensin II, p-aminophenylalanine-6 angiotensin II. *Biochem Biophys Res Commun* 1990;169:997–1006.
- [89] Steckelings UM, Wolschlagel T, Peters J, Henz BM, Hermes B, Artuc M. Human skin: source of and target organ for angiotensin II. *Exp Dermatol* 2004;13:148–54.
- [90] Steckelings UM, Henz BM, Wiestutz S, Unger T, Artuc M. Differential expression of angiotensin receptors in human cutaneous wound healing. *Br J Dermatol*, in press.
- [91] Stoll M, Hahn AW, Jonas U, Zhao Y, Schieffer B, Fischer JW, et al. Identification of a zinc finger homeodomain enhancer protein after AT2 receptor stimulation by differential mRNA display. *Arterioscler Thromb Vasc Biol* 2002;22:231–7.
- [92] Stoll M, Steckelings UM, Paul M, Bortner SP, Metzger R, Unger T. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest* 1995;95:651–7.
- [93] Sroth U, Meffert S, Gallinat S, Unger T. Angiotensin II and NGF differentially influence microtubule proteins in PC12W cells: role of the AT2 receptor. *Brain Res Mol Brain Res* 1998;53:187–95.
- [94] Summers C, Myers LM. Angiotensin II decreases cGMP levels in neuronal cultures from rat brain. *Am J Physiol* 1991;260:C79–87.
- [95] Thorup C, Kornfeld M, Winaver JM, Goligorsky MS, Moore LC. Angiotensin-II stimulates nitric oxide release in isolated perfused renal resistance arteries. *Pflügers Arch* 1998;435:432–4.
- [96] Tsutsumi K, Stromberg C, Viswanathan M, Saavedra JM. Angiotensin II receptor subtypes in fetal tissue of the rat: autoradiography, guanine nucleotide sensitivity, and association with phosphoinositide hydrolysis. *Endocrinology* 1991;129:1075–82.
- [97] Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, et al. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest* 1999;104:925–35.
- [98] Unger T. The angiotensin type 2 receptor: variations on an enigmatic theme. *J Hypertens* 1999;17:1775–86.
- [99] Wang ZQ, Moore AL, Ozono R, Siragy HM, Carey RM. Immunolocalization of subtype 2 angiotensin II (AT2) receptor protein in rat heart. *Hypertension* 1998;32:78–83.
- [100] Whitebread S, Mele M, Kamber B, de Gasparo M. Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Commun* 1989;163:284–91.
- [101] Widdop RE, Jones ES, Hannan RE, Gaspari TA. Angiotensin AT2 receptors: cardiovascular hope or hype? *Br J Pharmacol* 2003;140:809–24.
- [102] Wiemer G, Scholten BA, Wagner A, Heitsch H, Linn W. The possible role of angiotensin II subtype AT2 receptors in endothelial cells and isolated ischemic rat hearts. *J Hypertens Suppl* 1993;11(Suppl 5):S234–5.
- [103] Wruck CJ, Funke-Kaiser H, Pufe T, Kussow H, Menk M, Scheffé JH, et al. Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated Golgi protein. *Arterioscler Thromb Vasc Biol* 2005;25:57–64.
- [104] Wu L, Iwai M, Nakagami H, Chen R, Suzuki J, Akishita M, et al. Effect of angiotensin II type 1 receptor blockade on cardiac remodeling in angiotensin II type 2 receptor null mice. *Arterioscler Thromb Vasc Biol* 2002;22:49–54.
- [105] Wu L, Iwai M, Nakagami H, Li Z, Chen R, Suzuki J, et al. Roles of angiotensin II type 2 receptor stimulation associated with selective angiotensin II type 1 receptor blockade with valsartan in the improvement of inflammation-induced vascular injury. *Circulation* 2001;104:2716–21.
- [106] Zhang J, Pratt RE. The AT2 receptor selectively associates with Gialpha2 and Gialpha3 in the rat fetus. *J Biol Chem* 1996;271:15026–33.
- [107] Zhao Y, Biermann T, Luther C, Unger T, Culman J, Gohlke P. Contribution of bradykinin and nitric oxide to AT2 receptor-mediated differentiation in PC12 W cells. *J Neurochem* 2003;85:759–67.
- [108] Zhu YZ, Zhu YC, Li J, Schaefer H, Schmidt W, Yao T, et al. Effects of losartan on haemodynamic parameters and angiotensin receptor mRNA levels in rat heart after myocardial infarction. *J Renin Angiotensin Aldosterone Syst* 2000;1:257–62.

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT H

Introduction to Cytokines as Targets for Immunomodulation

Theresa L. Whiteside

SUMMARY

Cytokines play a key role in modulation of immune responses. Cytokine networks regulate lymphocyte turnover, differentiation, and activation. Many different cell types, in addition to immune cells, produce cytokines and express receptors for cytokines. Cell-to-cell communication (cellular "crosstalk") is maintained via cytokine networks. In disease, these networks undergo imbalance. By measuring amplification or downregulation of cytokine signaling cascades in response to pathological insults or therapeutic interventions, it might be possible to evaluate disease progression or regression. Multiplex formats for cytokine profiling are now available and appear to be especially useful in monitoring cytokine profile alterations in a variety of human diseases.

Key Words: Immune response; cytokine profiles; cytokines in disease; cytokines in therapy; cytokine crosstalk.

1. INTRODUCTION

In the last decade or so, cytokines, which are low molecular weight glycoproteins produced by immune as well as nonimmune cells, have captured the attention of the scientific and clinical communities. This unprecedented attention to cytokines can be, in part, attributed to their availability in a recombinant form, allowing for the use of individual cytokines in various experimental and clinical settings. Largely, however, it is because of the realization that complex cellular "soups" of the past (also referred to as "lymphokines") contain mixtures of defined soluble factors, cytokines, each of which plays a key role in orchestrating cellular interactions. The field of

From: Methods in Pharmacology and Toxicology: Cytokines in Human Health: Immunotoxicology, Pathology, and Therapeutic Applications
Edited by: R. V. House and J. Descotes © Humana Press Inc., Totowa, NJ

cytokine biology has emerged based on molecular, biochemical, and immunological insights into their structure, function, importance in health and disease, as well as therapeutic potential (reviewed in ref. 1) The number of cytokine genes cloned and individual cytokines identified has grown rapidly to include several distinct families of factors that include lymphokines, interferons, and hematopoietic and nonhematopoietic growth factors and appear to be necessary for cell-to-cell communication and signaling. Methods for the identification and measurements of the functional competence of cytokines have been developed that provide means for ascertaining their presence and following their levels of activity in body fluids and at tissues sites. The more recent discovery of chemokines, which are related to cytokines but remain structurally and functionally distinct (2), has further expanded the scope of soluble factors involved in the regulation of immunological responses, hematopoietic development, cell-to-cell communication, as well as host responses to infectious agents and inflammatory stimuli (3-6). To be able to fulfill this challenging assembly of biological activities, cytokines and chemokines are endowed with special biological characteristics that allow for effective functioning in the tissue microenvironment.

2. BIOLOGICAL CHARACTERISTICS OF CYTOKINES

The biology of cytokines is complex and, although new information emerges almost daily, much still remains to be learned. Cytokines share many characteristics with growth factors and hormones, such as platelet-derived growth factor, endothelial growth factor, or transforming growth factor- α . Although classical polypeptide hormones are produced by specialized cells, cytokines tend to be secreted by less-specialized cells, and often many different cell types produce the same cytokine. All cytokines are *pleiotropic*, i.e., they have the ability to interact not with one but a variety of cellular targets, usually via the specific receptors expressed on the cell surface. Expression of the receptor qualifies the cell as a target for one or more cytokines. Binding of cytokines to *cell surface receptors* (kDa in the range of 10^{-9} to 10^{-12} M) initiates signaling and new ribonucleic acid (RNA) and protein synthesis. Frequently, cytokine receptors are composed of several membrane-associated subunits, and one or more of these subunits might be shared by distinct cytokine receptors. Also, the presence or absence of individual subunits often determines the affinity of the cytokine receptor for its ligand as, for example, in the case of receptor for interleukin (IL)-2, in which the $\alpha\beta\gamma$ subunits are needed for high-affinity binding of IL-2, the $\beta\gamma$ sub-

units function as an intermediate affinity receptor and the α subunit is known as a low-affinity receptor for IL-2 (7). In addition, cytokines can modulate the level of receptor expression for another cytokine or growth factor, a phenomenon referred to as *receptor transmodulation*. For example, tumor necrosis factor (TNF)- α , IL-1, or IL-6 can affect IL-2 receptor expression (8-10), and interferon (IFN)- γ can modulate expression of TNF receptors on many different cell lines (11).

Cytokines are *redundant* in that the same biological function can be executed by several distinct cytokines. This overlapping of cytokine activities, whereby a single cell target shows seemingly identical responses to multiple cytokines, has been referred to as *cytokine crosstalk*. Cytokine redundancy is a biologically important feature because it provides a measure of safety for the process of regulation of normal cellular functions. Should one cytokine be absent or its level limited, an option for the substitution by another cytokine exists. Mechanistically, redundancy might be implemented via the shared receptor subunits or via the ability of different cytokine receptors to mediate similar signals, targeting the same molecular pathway(s). Physiologically, cytokine redundancy assures that the vital communication between cells participating in functions essential for survival continues uninterrupted regardless of the absence of one or another cytokine.

Cytokines and chemokines are known to function as *networks* or *cascades* of interacting factors, which are able to induce each other. This implies that cytokines *regulate their own production* via autocrine, juxtacrine, or paracrine pathways in response to microenvironmental stimuli. Cascades of cytokines are able to amplify the functional impact of one component and are related to functional redundancy. Thus, even when a redundant signal is weak, there is the possibility for its amplification via inducing the cascade of functionally related soluble factors. It is also essential to remember that local interactions and the state of activation of cells in the microenvironment are likely to have powerful effects on the cytokine production *in situ*.

Cytokines work in *pharmacological doses* and, once released by the producing cell, have a *short half-life*, often not exceeding a few minutes. This means that tiny quantities of cytokines present in the microenvironment for a short period of time are sufficient to induce functional changes in responding cells. Thus, cytokines are powerful physiological mediators with a potential for inducing *tissue damage*. Cytokines are *local mediators* meant to exert biological effects in their local microenvironment. Although in normal tissues, cytokine-mediated tissue damages do not occur, largely because of carefully regulated cytokine secretion, in pathological conditions, excessive cytokine release from activated cells not infrequently results in a local tissue

injury. Further, *systemic effects of cytokines* often are quite different and more dramatic than those mediated locally (12).

A division of labor exists among cytokines, in that the T-helper 1 (Th1)-type cytokines (e.g., IL-2, IFN- γ , IL-12, TNF- α) are considered to be responsible for elimination of intracellular infections, notably those caused by viruses or parasites, modulation of organ-specific autoimmune diseases, or mediating allograft rejection and recurrent abortions (13,14). Other human diseases are associated with Th2-type cytokine responses (e.g., IL-3, IL-4, IL-5, IL-13), including atopic disorders, chronic graft-versus host disease, progression to AIDS, cancer metastasis, or the hypereosinophilic syndrome (14). In general, the Th2-type cytokines are involved in promoting antibody-mediated responses. The third category of cytokines, Th3, includes regulatory mediators, such as IL-10 or TGF- β , which are responsible for maintaining a balance in the host microenvironment (15). This division of labor is a reflection of *cytokine polarization* and appears to be useful in the classification of certain human diseases into the Th1 or Th2 categories. It also forms a basis for a concept of the "cytokine profile." Because cytokine polarization occurs in many pathological conditions and cytokine cascades as well as cytokine crosstalk often are characteristically encountered in disease, the profiling of cytokines might prove to be more useful than measuring only one representative cytokine.

Cytokines are *polymorphic*, which means that genetic variability exists among individuals in terms of the ability to produce cytokines in response to a stimulus. Cytokine levels produced as a result of activation can vary widely between individuals, and this trend may be genetically controlled. However, most important, the existing levels of cytokines might determine susceptibility or resistance to disease (16). TNF- α and IL-10 levels, for example, appear to influence responses to and outcome of infectious diseases. Experimental evidence supports the concept that differences in susceptibility to and severity of diseases might be related to genetically defined differences among individuals to produce important cytokines. Thus, low TNF production was associated with a 10-fold risk of fatal outcome from meningococcal meningitis in humans, whereas high IL-10 levels were associated with a 20-fold greater risk of fatality (17,18). These observations have led to extensive examination of cytokine polymorphism and its disease associations and a search for genetic markers that best define such polymorphism (19).

Cytokines mediate interactions between a broad variety of cell types, and their dysregulated production might contribute to the disease pathogenesis. Under normal circumstances, no or only low levels of cytokines are detectable in body fluids or tissues. Therefore, their presence at elevated levels of expression indicates activation of cytokine pathways associated with

inflammation or disease progression. Cytokines associated with acute diseases are distinct from those detectable in chronic conditions. Certain cytokines may be present early, others late in the disease. Consistently observed associations of increased cytokine levels with a disease process or disease stage suggest that anticytokine therapy might be effective, at least in some cases. Therefore, an assessment of cytokine profiles in body fluids, tissues, or cells of patients with various diseases is important, particularly if cytokines themselves are used as therapeutic agents.

3. CYTOKINE FAMILIES

Structural differences among cytokines have made it possible to group them within "families" because primary sequences of certain cytokines show structural homology. For example, all members of the IFN- α/β family show at least 30% homology in their amino acid sequences (20). Such common structural features of cytokines permit their groupings into families, for instance, the IL-2/IL-4 family (IL-2, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor) or the TNF family (TNF- α , lymphotoxin- α , LT- β , FasL, CD40L, TRAIL, LIGHT, and others) or the IL-1 family (IL-1 α , IL-1 β , IL-1 receptor antagonist, IL-18). Structural features of cytokine receptors form the basis for further classification of cytokines into distinct groups, such as the "hematopoietin family receptors" or class I cytokine receptors and "interferon/IL-10 family receptors" or class II cytokine receptors (1). Cytokines signal via their receptors, and the understanding of the receptor structure, expression, and signal transduction is at the heart of cytokine biology. The unique features of the signaling pathways engaged by different cytokines and often associated with JAK and STAT pathways form the basis for cytokine crosstalk.

4. CYTOKINE PROFILES IN HUMAN DISEASE

Activation of a cytokine cascade by a physiological or pathological stimulus usually leads to the production and release of several biologically related cytokines. Thus, as indicated previously, IL-2, IFN- γ , and TNF- α production characterizes Th1-type immune responses, whereas IL-4, IL-5, and IL-10 are associated with Th2-type responses (13,14). In an immune response, the cytokine cascade is initiated by specific recognition of the antigenic stimulus by T-cell receptor, subsequent T-cell activation, and secretion of the cytokine elicited by the antigen. Amplification of this initiating signal may then result in a local cytokine cascade. An inflammatory event induces a coordinated release of several proinflammatory cytokines (3). After induction, cytokine concentrations may rapidly and differentially peak and then decrease to undetectable levels.

Today, a wide range of cytokine assays is available, providing researchers with an opportunity to evaluate the biology of cytokines and to establish their therapeutic potential (reviewed in ref. 21). Moreover, an improved understanding of cytokine interactions in the increasing numbers of pathological conditions has led to a consensus that the simultaneous assessment of many cytokines in a single biological sample, that is, "cytokine profiling," is a preferred approach (22). This change of paradigm in cytokine measurements was brought about by recent technical developments, and it is apparent that cytokine assays that best lend themselves to multiplex-type formats of the future slowly are replacing the traditional biological and immunological cytokine assays of the past (23).

Cytokine measurements in tissue or in the peripheral circulation have been an important part of the process of defining the role various cytokines play in health and disease. New multiplex formats for cytokine assays allow for precise quantification of many different cytokines in a single small volume of body fluid. It has been suggested long ago that local cytokine levels and activity are of considerably greater value for monitoring of pathological events in a target tissue than are systemic cytokine levels (24), with the notable exception of cytokine pharmacokinetics in subjects receiving cytokine therapies. However, tissue cytokine profiles remain largely unexplored, probably because of considerable difficulties in obtaining interstitial fluid. In this respect, a technique of microdialysis, using a commercially available biocatheter inserted into accessible tissue sites, proved to be an excellent approach to the recovery of multiple cytokines in a volume of approx 50 μ L of interstitial fluid (25). In our hands, microdialysis of oral mucosa in patients with HIV infection proved to be a powerful strategy to define the cytokine/chemokine profile in the milieu of viral entry, replication, and persistence (T. L. Whiteside and A. Rosenbloom, unpublished data). Combined with *in situ* immunostaining of mucosal biopsies to define intracellular expression of cytokines and cell surface expression of cytokine receptors, this strategy might prove to be more informative and more prognostically important than profiling of serum cytokines. In any event, a comparison between oral mucosa and peripheral blood cytokine profiles in normal volunteers and individuals with HIV at various stages of the disease is in progress to determine the extent of correlations or differences and their relationship to clinical end points.

Although significant progress has been made in linking certain cytokines to pathological changes in disease, interpretations of the results of cytokine profiling are not a trivial matter. Under normal conditions, basal levels of cytokines are low. In diseases such as cancer, chronic infectious diseases,

transplantation, or allergy, profiles of cytokines are beginning to emerge that might correlate to clinical end points. In cancer, advanced metastatic disease is associated with the Th2 cytokine profile. To characterize tumor-specific CD4⁺ T-cell responses in patients with cancer, single-cell assays, such as the enzyme-linked immunosorbent spot, can be used. Upon stimulation of peripheral blood CD4⁺ T-cells obtained from the peripheral circulation of patients with renal cell carcinoma with major histocompatibility class II-restricted peptides (e.g., HLA-DR4-restricted MAGE 6₁₂₁₋₁₄₄ peptide), a Th2-polarized response (IL-5 but not IFN- γ production) is elicited in patients with active disease (26). In contrast, disease-free patients (NED) respond by Th1-type cytokine production (IFN- γ and not IL-5). Furthermore, successful therapy in cancer patients is accompanied by a shift from the Th2 to Th1 cytokine polarization (26). Thus, it might be now possible to follow the frequency of peptide-reactive CD4⁺ T-cells in the patients' circulation before and after therapy to observe changes in their polarization profile and predict the success or failure of a therapeutic intervention based on the direction of this change. In patients with HIV-1, whose sera often contain viral cytokines and cytokine-receptor homologs that can bind to cellular receptors or to circulating cytokines, respectively, counteracting their biological function, the interpretation of cytokine assays may be very difficult (27). Nevertheless, significant shifts in the cytokine profiles in the course of infection and upon therapy with highly active antiretroviral therapy have been observed in patients with HIV (28). The newly emerging associations between the cytokine profiles and allergic diseases provide an example of how cytokine determinations contribute to a better understanding of disease pathogenesis (14,29,30). For example, T-cells and dendritic cells are considered to be central in the pathogenesis of psoriasis. These cells create a type 1 inflammatory pathway in the skin with an abundant release of IFN- γ , IL-23, and IL-12, which help sustain activation of this pathway (31). TNF- α and IFN- γ , which play a pivotal role in host defense against infections and in the development of Th1 responses, also are involved in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and type 1 diabetes (32). These and other observations suggest that serial monitoring of cytokine profiles during the course of disease or therapy might be clinically useful. At present, all these correlations are not quite robust enough to be able to consider cytokines or cytokine profiles as "biomarkers" of disease or disease activity. It is expected, however, that the application of genomics and proteomics to cytokine measurements will further facilitate efforts to monitor patients' plasma and/or tissues for levels of cytokines and to evaluate cytokine profiles for their utility as surrogate markers of disease.

5. CYTOKINES AND THE IMMUNE SYSTEM

Immune cells both depend on cytokines for development and function and, upon activation, produce a variety of cytokines. For these reasons, the ability of various lymphocyte or monocyte subsets to respond to and to produce cytokines in response to specific stimuli has been important in the understanding of the immune system. Lymphocyte homeostasis involves continuous maturation and differentiation of antigen-responsive cells and their turnover between the circulating pool and tissues (33). Lymphocyte survival depends on the growth factors and cytokines produced in various niches to which they migrate or home (34). Consequently, cytokine networks determine the lymphocyte turnover and orchestrate their differentiation into functional effector cells. Likewise, cytokines regulate differentiation of dendritic cell precursors into functional antigen-presenting cells (35). The fundamental operation of the immune system is thus cytokine driven, and thymic development of T-cells (36), B-cell differentiation into antibody-producing plasma cells (37), or maturation and activation of natural-killer cells (38) are all coordinated by distinct networks of cytokines. Once matured in the designated tissue microenvironment, immune effector cells expressing the required cytokine/chemokine receptor(s) migrate under the direction of chemotactic gradients and thus guided arrive at the target tissue to deliver appropriate signals (39). The latter are entirely dependent on the situation in the targeted microenvironment and can be amplified or inhibited by cytokines produced *in situ*, either by infiltrating immune cells themselves or tissue cells or most likely both cell types. This entire process can be initiated by a sudden alteration in the tissue microenvironment, such as a bacterial or viral infection, injury or death of cells. The remarkable sensitivity, rapidity and regulated amplification of all cytokine-mediated immune responses to tissue injury indicate that the cytokine network functions in tandem with or under control of the neuroendocrine system. Indeed, although cytokines have been thought of as hormones of the immune system, it is necessary to remember that nonimmune cells express cytokine receptors and produce cytokines. Thus, cytokines are ubiquitous and appear to be able to maintain communications between cells of different origin and different systems. For example, it is now well documented that interactions among the central nervous system (CNS), endocrine networks, and immune cells determine the overall immune response. The CNS influences immune responses through the release of humoral factors such as cortisol or epinephrine from the hypothalamic-pituitary-adrenal axis. Lymphoid organs, e.g., lymph nodes, are innervated, and local synthesis of neuropeptides or opioids in these nerve terminals has

been documented. Immune cells express surface receptors for neurotransmitters, and nerve fibers have receptors for cytokines (40). Thus, a bidirectional traffic of biochemical mediators, cytokines, and neuropeptides between the CNS and immune cells regulates their interactions. When glucocorticoids are released in response to systemic stress, they downregulate immune responses and cytokine production. Migration inhibitory factor, a cytokine produced by the pituitary, counteracts inhibitory effects of glucocorticoids and restores the immune balance (41). Hence, cytokines play a key role in regulating the neuroendocrine-immune system interface. The mechanisms or molecular pathways involved in these interactions are now being elucidated. The field of neuroimmunology has grasped the likely importance of cytokines in the CNS responses, and much current work is devoted to studies of these interactions in animal models of CNS injury or stress as well as in human models of acute or chronic stress (42).

6. IMMUNOMODULATION BY CYTOKINES IN THERAPY OF HUMAN DISEASES

Ever since recombinant cytokines became available more than 20 years ago, numerous phase I and phase II clinical trials have been performed with these agents worldwide. By and large, these trials have capitalized on preclinical *in vitro* and *in vivo* animal model studies demonstrating immunomodulatory effects of cytokines on the host immune system and its components. To date, IL-2 and IFN- α are the only cytokines approved by Food and Drug Administration as therapies for renal cell carcinoma and melanoma, respectively. IFN- α is also approved for therapy of hepatitis C and hairy cell leukemia (43). Nevertheless, the therapeutic potential of many other cytokines is being evaluated in experimental clinical settings.

From the start, two opposite strategies for cytokine-based therapy targeting immune cells have emerged and still exist today. In one, a therapeutic cytokine of choice is systemically administered at very high doses to a subject with defects in the immune system. The objective is to provide the exogenous cytokine in excess to "force" immune recovery and induce a clinical response. The high-dose systemic delivery of cytokines is invariably accompanied by severe toxicity, which may be life threatening (e.g., resulting in a vascular leak syndrome upon high-dose IL-2 therapy) and usually is accompanied by a variety of side effects (44). Although this form of cytokine therapy appears to be nonphysiological and not in agreement with basic principles of the cytokine biology, surprisingly, it has been successful in a limited number of cases (45). The molecular underpinnings of such high-dose IL-2 or IFN- α therapies are not clear, but it has been suggested that cascades

of other cytokine (e.g., $\text{TNF-}\alpha$ or $\text{IL-1}\beta$) are responsible for toxicity as well as restoration of immune cell numbers or functions reported in many instances (46). The second approach depends on locoregional delivery of the cytokine to the targeted organ or site (e.g., tumor site, infection site) with the hope of increasing immune cell numbers and augmenting effector cell functions *in situ*. Locoregional delivery of cytokines does not induce severe toxicities, it is usually well tolerated, and it has been shown to alter immune cell infiltration and activation *in situ* (47). This form of cytokine therapy is based on a rationale that cytokines are local mediators meant to function at short distances in tissue, and although it is appropriate for therapy of local diseases, its usefulness in treatment of the systemic disease might be limited. Still, it should be noted that local, for instance, peritumoral or perilymphatic, delivery of cytokines has been shown to induce local as well as systemic changes in immune cell activation (47). Numerous and clever ways of cytokine delivery to target tissues have been introduced, including microspheres, liposomes, viral and nonviral vectors, or plasmids. These strategies, many of which are discussed in this volume, are meant to assure continuous release of the mediator in hope of inducing persistent activation of immune cells in the microenvironment. In animal models of disease, these strategies have been successful in inducing long-lasting and protective changes in the host immune system (48). Their application to therapy of human disease is in progress, pending safety and toxicity considerations. At present, experimental preclinical and clinical studies using either systemic or local cytokine delivery are ongoing in various institutions, and expectations are that immune monitoring and clinical results will help in defining mechanisms responsible for documented objective responses in each of these settings.

A considerable controversy exists in regard to therapeutic delivery of single recombinant cytokines vs natural mixtures of cytokines. Again, two widely held views conflict in that one favors therapy with well-defined, precisely dosed single cytokines or possibly a combination of selected recombinant cytokines, whereas the other insists that naturally derived cytokine mixtures, for instance, partially purified supernatants of mitogen-activated lymphocytes, are more effective in upregulating a variety of immune effector cells and thus providing a broader therapeutic index (49). Although there are advantages and disadvantages to each of these approaches, the controversy emphasizes the need for further studies of cytokines as immunomodulators that potentially are capable of correcting pathological conditions.

Finally, excessive production of cytokines has been associated with a spectrum of human diseases, including posttransplant rejection episodes. In

all these conditions, the control of cytokine cascades or inhibition of individual cytokines are desirable. Therapeutic strategies that attempt to restore the cytokine balance disturbed by pathological overproduction of cytokines include systemic or local delivery of inhibitory cytokines, antibodies to cytokines, antibodies or agents blocking cytokine receptors, and *in situ* modifications of cytokine genes, for example, with inhibitory RNA among others. The difficulties associated with these approaches relate to the fact that little is known about the regulatory mechanisms of cytokine production *in vivo* and that *in vitro* models of these molecular pathways in cell lines do not adequately reflect cellular controls operative in tissues.

Overall, cytokine-based therapies alone or in combination with other drug modalities are considered to be promising and are continued to be evaluated for treatment of various human diseases. Many novel therapeutic strategies involving cytokine delivery and monitoring of cytokine responses *in vivo* have been introduced in recent years. The use of fusokines or multifunctional cytokines representing fusion proteins linking two cytokines and expressing them in an expression system convenient for *in vivo* delivery represent one such strategy (50). Immunocytokines are fusion proteins combining a cytokine and an antibody (51). They have been shown to have potent antitumor effects and are under intensive experimental scrutiny at this time. There are reasons to believe that, in years to come, cytokine-based therapies might fulfill their promise and become a part of the widely applicable therapeutic armamentarium for many human diseases.

7. CONCLUSIONS

The role of cytokines and chemokines in immunomodulation has been well established and generally is considered to be the basis for their biological and therapeutic effects. Various immune cell subsets express receptors for cytokines, produce cytokines upon activation and are functionally dependent on cytokines. However, immunomodulatory effects of cytokines/chemokines are superseded by their role in networking functions of immune as well as non-immune cells. Cytokines bridge and regulate activities of many different cell types. Their participation in functions of the CNS, endocrine, reproductive, hematopoietic, and immune systems places cytokines at the interface and indicates that they are ubiquitous and essential for survival. Their biological characteristics and therapeutic potential further support this notion. A rapidly expanding body of recent evidence indicates that cytokines are essential for cell-to-cell communication and for orchestrating local cellular events in a variety of tissues. As such, they subserve a central role of linking and integrating signals delivered by different systems. For

this reason, the understanding of cytokine crosstalk and their signaling at the molecular level are special challenges to be faced by investigators in the near future. Only through such an understanding are we likely to be able to harness cytokines/chemokines as useful pharmacological and therapeutic agents for human diseases.

ACKNOWLEDGMENTS

Supported in part by grants PO1-DE12321, RO1-CA82016 and RO1-DE13918 from National Institutes of Health.

REFERENCES

1. Vilcek J. The cytokines: an overview. In: Thomson AW, Lotze MT, eds. *The Cytokine Handbook*. 4th ed, Boston, Academic Press, 2003, pp. 3–18.
2. Zlotnik A, Osamu Y. Chemokines: a new classification system and their role in immunity. *Immunity* 2000;12:121–127.
3. Dinarello CA, Kluger MJ, Powanda MC. *The Physiological and Pathological Effects of Cytokines*. New York: Liss; 1990.
4. Oppenheim JJ. *Clinical Applications of Cytokines. Role in Pathogenesis, Diagnosis and Therapy*. New York: Oxford University Press; 1993.
5. Aggarwal BB, Puri RK, eds. *Human Cytokines: Their Role in Disease and Therapy*. Cambridge, MA: Blackwell Science; 1995.
6. Meager T. *The Molecule Biology of Cytokines*. Chichester: John Wiley & Sons; 1998.
7. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. *Cell* 1993;11:245–267.
8. Smith KA. Interleukin-2: inception, impact, and implications. *Science* 1988;240:1169–1176.
9. Hatakeyama M, Tsudo M, Minamoto S, et al. Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNAs. *Science* 1989;244:551–556.
10. Lowenthal JW, Ballard DW, Bogerd H, Bohnlein E, Greene WC. Tumor necrosis factor- α activation of the IL-2 receptor- α gene involves the induction of κ B-specific DNA binding proteins. *J Immunol* 1989;142:3121–3128.
11. Aggarwal BB, Eessalu TE, Hass PE. Characterization of receptors for human tumour necrosis factor and their regulation by γ -interferon. *Nature* 1985;318:665–667.
12. Dinarello CA, Wolff SM. Mechanisms of disease: the role of interleukin 1 in disease. *N Engl J Med* 1993;328:106–113.
13. Romagnani, S. The Th1/Th2 paradigm. *Immunol Today* 1997;18:263–266.
14. Romagnani, S. *The Th1/Th2 Paradigm in Disease*. Austin: R.G. Landes Co.; 1997.
15. Mosmann TR, Li L, Sad S. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin Immunol* 1997;9:87–92.

16. Sorensen TT, Nielsen GG, Andersen PK, Teasdale TW. Genetic and environmental influences on premature death in adult adoptees. *N Engl J Med* 1988; 318:727-732.
17. Westendorp RG, Langermans JA, Huizinga TW, Verweij CL, Sturk A. Genetic influence on cytokine production in meningococcal disease. *Lancet* 1997;349:1912-1913.
18. van Dissel JT, van Langevelde P, Westendorp RG, et al. Anti-inflammatory cytokine profile and mortality in febrile patients. *Lancet* 1998;351:950-953.
19. Gallagher G, Eskdale J, Bidwell JL. Cytokine genetics - polymorphisms, functional variations and disease associations. In: Thomson AW, Lotze MT, eds. *The Cytokine Handbook*, 4th ed. Boston: Academic Press; -2003, pp. 19-55.
20. Langer JA, Pestka S. Interferon receptors. *Immunol Today* 1989;12:393-400.
21. Whiteside TL. Assays for Cytokines. In: Thomson AW, Lotze MT, eds. *The Cytokine Handbook*, 4th ed. Boston: Academic Press; 2003, pp. 1375-1396.
22. De Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *CDLI* 2003;10:133-139.
23. Whiteside TL. Cytokine assays. *Biotechniques/Cytokines* 2002;33:4-15.
24. Mathey E, Pollard J, Armati P. *In situ* hybridization for cytokines in human tissue biopsies. *Methods Mol Biol* 2003;204:57-66.
25. Kjellstrom S, Appels N, Ohlrogge M, Laurell T, Marko-Varga G. Microdialysis—a membrane based sampling technique for quantitative determination of proteins. *J Chromatogr A* 1999;50:539-546.
26. Tatsumi T, Kierstead LS, Ranieri E, et al. Disease-associated bias in T helper type 1 (Th1)/Th2 CD4+ T-cell responses against MAGE-6 in HLA-DRB1*0401+ patients with renal cell carcinoma or melanoma. *J Exp Med* 2002;196:619-628.
27. Nicholas J, Ruvoilo VR, Burns WH, et al. Kaposi's sarcoma-associated human herpesvirus-8 encodes homologues of macrophage inflammatory protein-1 and interleukin-6. *Nat Med* 1997;3:287-292.
28. Connor RI, Sheridan KE, Ceradini D, Choe S, Landau N. Change in coreceptor use correlates with disease progression in HIV-1 infected individuals. *J Exp Med* 1997;185:621-628.
29. Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infections, neoplastic and inflammatory diseases. *Clin Microbiol Rev* 1996;9:532-562.
30. Grewe M, Bruijnzeel-Koomen CA, Schopf E, et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 1998;19:539-361.
31. Lew W, Bowcock AM, Krueger JG. Psoriasis vulgaris: cutaneous lymphoid tissue supports T-cell activation and "Type 1" inflammatory gene expression. *Trends Immunol* 2004;25:295-305.
32. O'Shea JJ, Ma A, Lipsky P. Cytokines and autoimmunity. *Nat Rev Immunol* 2002;2:37-45.

33. Surh CD, Sprent J. Regulation of naïve and memory T cell homeostasis. *Microbes Infect* 2000;4:51–56.
34. Khaled A, Durum SK. The role of cytokines in lymphocyte homeostasis. *Biotechniques* 2002;33:540–545.
35. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony stimulating factor plus interleukin-4 and down-regulated by tumor necrosis factor alpha. *J Exp Med* 1994;179:1109–1118.
36. Schluns KS, Lefrançois L. Cytokine control of memory T-cell development and survival. *Nat Rev* 2003;3:269–279.
37. Kishimoto T. Factors affecting B cell growth and differentiation. *Annu Rev Immunol* 1985;3:133–157.
38. Biron C. Activation of natural killer cell responses during viral infections. *Curr Opin Immunol* 1997;9:24–34.
39. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nat Immunol* 2001;2:123–128.
40. Haddad JJ, Saade NE, Safieh-Garabedian B. Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis. *J Neuroimmunol* 2002;133:1–19.
41. Calandra T, Bucala R. Macrophage migration inhibition factors MIF: a glucocorticoid counter regulator within the immune system. *Crit Rev Immunol* 1997;17:77–88.
42. Straub RH, Westermann J, Scholmerich J, Falk W. Dialogue between the CNS and the immune system in lymphoid organs. *Immunol Today* 1998;19:409–413.
43. Foon KA, Maluish AE, Abrams PG, et al. Recombinant leukocyte A interferon therapy for advanced hairy cell leukemia: therapeutic and immunological results. *Am J Med* 1986;80:351–356.
44. Baluna R, Vitetta ES. Vascular leak syndrome: a side effect of immunotherapy. *Immunopharmacology* 1997;37:117–132.
45. Rosenberg SA, Lotze MT, Yang JC, et al. Experience with the use of high dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg* 1989;210:474–485.
46. Kirkwood JM, Richards T, Zarour HM, et al. Immunomodulatory effects of high-dose and low-dose interferon $\alpha 2b$ in patients with high-risk resected melanoma. *Cancer* 2002;95:1101–1112.
47. Whiteside TL, Letessier E, Hirabayashi H, et al. Evidence for local and systemic activation of immune cells by peritumoral injections of interleukin 2 in patients with advanced squamous cell carcinoma of the head and neck. *Cancer Res* 1993;53:5654–5662.
48. Pardoll DM. Paracrine cytokine adjuvants in cancer immunotherapy. *Annu Rev Immunol* 1995;13:399–415.
49. Meneses A, Verastegui E, Barrera JL, DeGarz J, Hadden JW. Lymph node histology in head and neck cancer: impact of immunotherapy with IRX-2. *Int Immunopharmacol* 2003;3:1083–1091.

50. Gillies SD, Lan Y, Brunkhorst B, Wong WK, Li Y, Lo KM. Bifunctional cytokine fusion proteins for gene therapy and antibody-targeted treatment of cancer. *Cancer Immunol Immunother* 2002;51:449–460.
51. Lode HN, Xiang R, Becker JC, Gillies SD, Reisfeld RA. Immunocytokines: a promising approach to cancer immunotherapy. *Pharmacol Ther* 1998;80:277–292.

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT I

New regulators of NF- κ B in inflammation

Sankar Ghosh*† and Matthew S. Hayden*

Abstract | Research on the biological function of nuclear factor- κ B (NF- κ B), a key mediator of inducible transcription in the immune system, has traditionally focused on its role in the initiation of innate and adaptive immune responses. These studies have largely concentrated on the mechanisms of signalling that lead to NF- κ B activation and on the positive role of NF- κ B in both physiological immunity and pathological inflammation. More recently, there has been growing interest in the mechanisms that directly regulate the NF- κ B transcriptional programmes. As a result, several new NF- κ B regulatory components have been identified and some of the known components have been assigned new roles. In this Review, we discuss these new insights into the regulation of NF- κ B.

LPS tolerance
A transient state of hyporesponsiveness to subsequent stimulation with the Toll-like receptor (TLR) ligand LPS following previous exposure to TLR ligands.

The mammalian nuclear factor- κ B (NF- κ B) family consists of five related transcription factors that regulate inducible gene expression in various physiological contexts. The link between the activation of NF- κ B and inflammation has been shown in various human diseases and in animal models of disease. In addition, the role of NF- κ B in mediating inflammation has been established using genetic approaches or with chemical inhibitors. In most cases, either epithelial cells of the infected tissue or tissue-resident haematopoietic cells, such as mast cells or dendritic cells (DCs), initiate the inflammatory response by triggering pro-inflammatory pathways through NF- κ B in response to inflammatory stimuli¹. Activation of NF- κ B results in the upregulation of adhesion molecules and chemokines by vascular endothelial cells and within the tissue. This then leads to the recruitment and activation of effector cells, initially neutrophils and then macrophages and other leukocytes. NF- κ B is also crucial for the production of antimicrobial effector molecules and for the survival of leukocytes in an inflammatory milieu. In fact, NF- κ B has a fundamental role in mediating all of the classical attributes of inflammation — rubor, calor, dolor and tumor — by regulating transcriptional programmes in tissue epithelial and stromal cells, vascular endothelial cells and haematopoietic cells.

The pro-inflammatory transcriptional programmes that are regulated by NF- κ B organize and execute the inflammatory response. These transcriptional programmes differ depending on the inducing stimulus and on the cell type that is mediating the response. Therefore, the NF- κ B-dependent genes that are expressed by

endothelial cells in response to the microbial product lipopolysaccharide (LPS) can be different from those that are induced in response to the pro-inflammatory cytokine tumour-necrosis factor (TNF). Although some of these differences arise from epigenetic modifications that are associated with different genes in different cell types, there is little doubt that a significant degree of diversity results from the complexity of the NF- κ B system. Indeed, even repeated stimulation of the same cells with the same stimuli can result in a markedly altered transcriptional output. For example, it has recently been shown that in LPS tolerance only the NF- κ B-regulated pro-inflammatory genes that could be harmful if continually expressed are hyporesponsive to repeated LPS stimulation, and are therefore tolerizable genes. By contrast, other NF- κ B-regulated genes, such as antimicrobial peptides, are non-tolerizable², as their continued expression is necessary for protection against infection.

The established model of NF- κ B signalling holds that, in unstimulated cells, inhibitor of NF- κ B (I κ B) proteins sequester the inactive transcription factor in the cytoplasm (Fig. 1). In this model, the key regulatory event in NF- κ B activation is the phosphorylation of I κ B proteins by the I κ B kinase (IKK) complex, which leads to I κ B protein ubiquitination and subsequent degradation. This results in the release of cytoplasmic NF- κ B complexes, which then translocate to the nucleus and drive the expression of target genes. Therefore, the signalling pathways that lead to the activation of the IKK complex are crucial regulatory events in NF- κ B activation. However, studies carried out over the past 10 years have revealed a far more complicated picture of how NF- κ B is activated. We now know

*Department of Immunobiology and
†Department of Molecular Biophysics and Biochemistry,
Yale University School of Medicine, New Haven, Connecticut 06510, USA.
Correspondence to S.G.
e-mail: sankar.ghosh@yale.edu
doi:10.1038/nri12423
Published online 17 October 2008

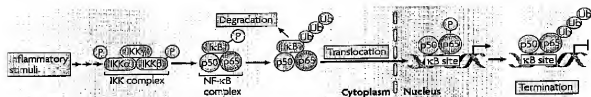


Figure 1 | Relevant regulatory nodes in NF- κ B signalling pathways. In the simplest model of nuclear factor- κ B (NF- κ B) activation, an inflammatory stimulus activates signal transduction pathways that induce the activation of the IKK (inhibitor of NF- κ B (κ B) kinase) complex. This results in the phosphorylation of I κ B proteins and consequently their degradation by the proteasome. Released NF- κ B dimers translocate to the nucleus and bind κ B sites in the promoters or enhancers of target genes, which leads to their transcription. Each step represents a regulatory node with several known inputs and outputs. These include additional IKK targets that affect NF- κ B transcriptional activity, the role of I κ B proteins in shaping transcriptional programmes, signalling pathways and cofactors that lead to selective gene expression by NF- κ B, and mechanisms that control the termination of NF- κ B activity. Additional regulatory events in NF- κ B pathways, including negative feedback loops, are not shown. Note that the green phosphate signifies phosphorylation that results in negative regulation of the phosphorylated protein, whereas the yellow phosphate indicates activation. Ub, ubiquitin.

that there is a complex interplay between signal-induced post-translational modifications of NF- κ B and the ability of this transcription factor to both promote and inhibit the transcription of target genes.

Understanding the intricate relationships between NF- κ B signalling pathways and the variability in the expression pattern of NF- κ B-regulated genes is currently a key focus in the field of NF- κ B signalling research. The ability to effectively target NF- κ B for therapeutic intervention requires an understanding of how these intricacies govern NF- κ B activity. What regulates selectivity in the activation of NF- κ B transcriptional programmes? What terminates NF- κ B responses and how is this process controlled? Answers to such questions will probably require the identification of new regulatory components and new functions for known components of the NF- κ B pathway. In this Review, we discuss recent insights into the functions of IKK and I κ B proteins, describe new intermediates that shape the NF- κ B transcriptional response and consider new mechanisms that are responsible for terminating NF- κ B transcriptional activity. Of course, there are many interesting regulators of NF- κ B, new and old, that fit this description and are not discussed here — most of these have been reviewed extensively in REFS 3–9. In this Review, we focus on factors that we believe reveal new mechanisms of selective regulation of NF- κ B.

Components of the NF- κ B signalling pathway

The mammalian NF- κ B family consists of p50 (also known as NF- κ B1), p52 (also known as NF- κ B2), p62 (also known as REL λ), REL and REL β , which all share an amino-terminal REL homology domain (RHD) and are regulated by eight I κ B family members (FIG 2; TABLE 1). Dimers of NF- κ B proteins bind to κ B sites in promoters or enhancers of target genes, the transcription of which is regulated through the recruitment of transcriptional co-activators and transcriptional co-repressors². Transcription of p65, REL β and REL is positively regulated through the transactivation domain (TAD). By contrast, p50 and p52 lack TADs and therefore can function in three possible ways: by altering κ B-site specificity as heterodimers with TAD-containing family member; by repressing

transcription when bound to κ B sites as homodimers, or by promoting transcription through the recruitment of other TAD-containing proteins to the κ B sites.

In unstimulated cells, NF- κ B is sequestered in the cytoplasm by I κ B proteins. The I κ B protein family comprises three functional groups: the typical I κ B proteins I κ B α , I κ B β and I κ B ϵ , which are present in the cytoplasm of unstimulated cells and undergo stimulus-induced degradation and resynthesis; the precursor proteins p100 and p105, which can be processed to form the NF- κ B family members p52 and p50, respectively, or can be degraded; and the atypical I κ B proteins I κ B δ (encoded by *NFKBIZ*), BCL-3 (B-cell lymphoma 3) and I κ BNS (encoded by *NFKBID*), which are generally not expressed in unstimulated cells, but are induced following activation and mediate their effects in the nucleus. I κ B α is the prototypical member of the I κ B family of proteins. Following cell stimulation, it undergoes rapid ubiquitin-mediated proteasomal degradation that results in the release of the bound, cytoplasmic NF- κ B dimers. Cytoplasmic NF- κ B then translocates to the nucleus and drives gene expression, including that of I κ B α , which facilitates the termination of the transcriptional response by binding and retaining the NF- κ B dimers in the cytoplasm⁴. The dominance of I κ B α in both the rapid release of the prototypical p50–p55 NF- κ B dimer and in the negative regulation of NF- κ B has slowed efforts to understand the unique functions of other I κ B family members. However, recent studies have begun to reveal the importance of atypical members of the I κ B family (see later).

The IKK complex, which phosphorylates I κ B following activation, consists of two homologous kinase subunits, IKK α (also known as IKK1) and IKK β (also known as IKK2), and a regulatory subunit, NEMO (also known as IKK γ). In most canonical NF- κ B signalling pathways (including those that are activated by the pro-inflammatory cytokines TNF and interleukin-1 (IL-1), and by various microbial products), IKK β is necessary and sufficient for the phosphorylation of I κ B α , although there is increasing evidence of a role for IKK α in this process¹⁰. Even when IKK α is dispensable for I κ B α phosphorylation, it can function as an important regulator of the NF- κ B-dependent transcriptional

Transcriptional co-activator
A protein complex that associates with the ligand-binding domain of peroxisome-proliferator-activated receptors (PPARs) and other transcription factors. Transcriptional co-activators reorganize chromatin templates and recruit the basal transcriptional machinery to the promoter region.

Transcriptional co-repressor
A protein complex (including N-CoR and SMRT) that recruits histone-deacetylase complexes to reverse the actions of histone acetyltransferases and that inhibits gene transcription when associated with nuclear hormone receptors.

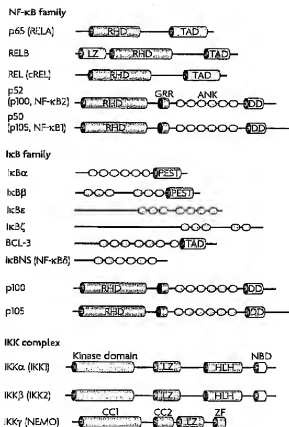


Figure 2 | Members of the NF- κ B, I κ B and IKK protein families. The domains that typify each protein family are indicated, and alternative nomenclatures are provided in brackets. The precursor proteins p100 and p105 function as both I κ B (inhibitor of nuclear factor- κ B (NF- κ B)) proteins and, when processed by the proteasome, NF- κ B family members. ANK, ankyrin-repeat; BCL-3, B-cell lymphoma 3; CC, coiled-coil; DD, death domain; GRR, glycine-rich region; HLH, helix-loop-helix; IKK, I κ B kinase; LZ, leucine zipper; NBD, NF- κ B-essential-modulator-binding domain; PEST, proline- glutamic acid-, serine- and threonine-rich; RHD, REL homology domain; TAD, transactivation domain; ZF, zinc-finger.

response through non-I κ B substrates, such as NF- κ B itself, transcriptional co-activators and transcriptional co-repressors¹⁴. IKK γ is required for all canonical NF- κ B signalling pathways, including those that are catalysed by IKK α . By contrast, the non-canonical NF- κ B pathway proceeds through the induction of p100 processing and the release of p52-containing dimers, and this process is thought to depend primarily on IKK α . The non-canonical pathway is activated mainly through members of the TNF receptor superfamily, such as the lymphotxin- β receptor^{14,15}.

Negative feedback loops that involve activating and inhibitory phosphorylation events are well described in several NF- κ B pathways. More recently, an analogous role has been proposed for the post-translational ubiquitination of signalling intermediates¹⁶. However, relatively less effort has been devoted to examining the regulatory mechanisms that impinge directly on

the transcriptional activity of NF- κ B. Historically, this oversight probably stems from the fact that NF- κ B-induced I κ B α expression provides a robust and dominant negative feedback loop that is directed against NF- κ B-dependent transcriptional activity.

IKK α : more than just an I κ B kinase

In addition to the main function of both IKK α and IKK β , which is the phosphorylation of I κ B proteins and the subsequent release of NF- κ B dimers (FIG. 3a), both IKK α and IKK β have been implicated in the direct phosphorylation of NF- κ B proteins, in particular of the p65 subunit¹⁷ (FIG. 3b). This phosphorylation can regulate the recruitment of essential transcriptional co-activators and co-repressors to NF- κ B in the nucleus, thereby positively influencing NF- κ B-dependent gene expression¹⁸. Intriguingly, however, IKK α -deficient mice show higher inflammatory responses than wild-type mice, which suggests that IKK α has a role in restricting the transcription of some NF- κ B-regulated genes¹⁹. The mechanism of IKK α -mediated repression of transcriptional responses seems to be mediated by alterations in the expression levels of nuclear p65 and REL²⁰ (FIG. 3c). Similar results were seen in embryonic-liver-derived macrophages from IKK α -deficient mice, which expressed high levels of several NF- κ B-regulated pro-inflammatory genes²¹. However, there are significant differences between these two reports. Li *et al.* observed a modest increase in the kinase activity of IKK β in IKK α -deficient macrophages, which they attributed to decreased I κ B α expression levels and increased NF- κ B transcriptional activity²¹, whereas Lawrence *et al.* did not observe the same effect with a knock-in mouse model expressing a mutant form of IKK α that lacked kinase activity¹⁹. Conversely, the loss of p65 phosphorylation and the decrease in p65 and REL degradation that was observed by Lawrence *et al.* was not seen by Li *et al.* These differences could be due to the use of mice that lacked IKK α compared with the use of mice that expressed IKK α with a point mutation, and to the type of macrophage used in the studies. Regardless of these differences, a functional role for IKK α in limiting pro-inflammatory macrophage responses was observed in both studies.

The proposed role for IKK α in regulating the expression levels of nuclear p65 and REL is not the only nuclear function that has been suggested for this protein kinase. IKK α may target both transcriptional co-activators and co-repressors, and may bind to the promoters of NF- κ B-regulated genes^{14,15}. It was initially proposed that promoter-bound IKK α was responsible for inducing the phosphorylation of serine 10 in histone H3 (REFS 14, 15); however, whether this is a direct or indirect effect is still unclear (FIG. 3d). More recently, IKK α was shown to induce derepression of the SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) co-repressor complex through direct phosphorylation, which facilitates opening up of the chromatin structure, and subsequent binding and transactivation by NF- κ B¹⁶ (FIG. 3e). Phosphorylation of SMRT by IKK α induces the displacement of histone deacetylase 3 (HDAC3) from SMRT complexes that

Table 1 | Function of I κ B family proteins in NF- κ B activation

Protein	Function in NF- κ B signalling	Effect on transcription	Refs
I κ B α	Binds free NF- κ B, promotes cytoplasmic localization	↓	4,7,8
I κ B β	Binds free NF- κ B	↓ or unknown	4,7,8
I κ B ϵ	Binds free NF- κ B, promotes cytoplasmic localization	↓	4,7,8
BCL-3	Phosphorylated BCL-3 binds DNA that is bound to p50 and p52 homodimers	↑	23,24
	Deubiquitinated BCL-3 sequesters p50 in the cytoplasm	↓	27
	Protects p50 homodimers bound to DNA from ubiquitination	↓	31
I κ B ζ	Binds DNA that is bound to p50 homodimers	↑	35,36
I κ BNS	Binds DNA that is bound to p50 homodimers	↑	37,38,39
p100	Processed to p52 subunit	↑	4,7,8
	Binds p52-RELB complexes	↓	45
	Binds RELB, processed to p52 in alternative pathway	↑	42,45
	Binds and inhibits free NF- κ B dimers	↓	52
p10S	Processed to p50 subunit	↑	4,7,8
	Binds and inhibits free NF- κ B dimers, particularly p50-containing homodimers, and promotes cytoplasmic localization	↓	4,7,8

↓ denotes a decrease in transcription and ↑ denotes an increase. For functions that are not discussed in the text, see REFS 4,7,8. BCL-3, B-cell lymphoma 3; I κ B, inhibitor of nuclear factor- κ B (NF- κ B).

are bound to p50 homodimers³⁷. This loss of repression and the resulting opening of the surrounding chromatin structure facilitate the replacement of the p50 homodimers with transcriptionally active NF- κ B dimers. Therefore, IKK α may increase transcription by indirectly promoting histone acetylation and DNA remodelling, or by increasing the acetylation and transcriptional activity of p53 (REF. 6). As SMRT co-repressor complexes are shared between many transcription factors, it is unclear whether IKK α selectively affects NF- κ B-regulated transcription. Indeed, phosphorylation of SMRT by IKK α may also affect transcriptional events that are induced by Notch³⁸. However, the mechanism (or mechanisms) that targets IKK α specifically to certain promoters remains to be clarified.

Although IKK α -induced phosphorylation of SMRT and removal of HDAC3 would relieve repression, recruitment of an active histone acetyltransferase (HAT) complex is also necessary for productive gene expression. IKK α may also increase this aspect of the NF- κ B transcriptional response. More specifically, IKK α can directly phosphorylate CBP (CREB-binding protein) at serine 1382 and serine 1386, which leads to enhanced binding to p65 (REF. 19) (FIG. 5c). The preferential binding of p65 to CBP rather than to the tumour suppressor transcription factor p53 that results from phosphorylation of CBP by IKK α is one possible mechanism by which p53 and NF- κ B may cross-regulate each other³⁹. This suggests that by facilitating aspects of the NF- κ B response, IKK α may repress transactivation by other transcription factors. Recently, activation of IKK α by RANKL (receptor activator of NF- κ B ligand) was shown to inhibit the transcription of the tumour suppressor Maspin (also known as SERPINB5)⁴⁰, expression of which is not regulated by NF- κ B dimers. However,

IKK α associates with the Maspin promoter, which suggests that IKK α can repress gene expression independently of NF- κ B⁴⁰. Interestingly, expression of Maspin is regulated by p53 (REF. 21), which raises the possibility that IKK α phosphorylates CBP and thereby disrupts its binding to the Maspin promoter. This would lead to reduced Maspin expression and would promote the expression of NF- κ B-dependent genes by facilitating CBP binding to active NF- κ B complexes.

Therefore, IKK α seems to be a remarkably versatile molecule that can function not only in its traditional role as a kinase for I κ B, but also in other ways that affect gene expression (FIG. 3). This surprising complexity of the biological role of IKK proteins suggests that the therapeutic use of IKK inhibitors will be less straightforward than initially anticipated, but also offers hope that selective inhibition of IKK α or IKK β could provide more control over NF- κ B responses than previously would have been thought.

Atypical I κ B proteins and inflammation

The suggested function of I κ B proteins has evolved in recent years from being simple inhibitors of NF- κ B activity to more complex regulators of gene expression (FIG. 4). For example, I κ B proteins can promote the formation of otherwise unstable NF- κ B dimers, such as RELB-containing dimers, and thereby influence the range of NF- κ B dimers that are present in a given cell. In addition to sequestering NF- κ B dimers in the cytoplasm, I κ B family members have also been suggested to stabilize nuclear and DNA-bound dimers. Moreover, some I κ B proteins may positively regulate transcription by acting as transcriptional co-activators. Therefore, instead of considering I κ B proteins solely as inhibitors of NF- κ B, it is probably more accurate to consider them as pleiotropic NF- κ B cofactors. Here,

Notch

A transmembrane receptor that is involved in the pathway for direct cell-cell signalling through its association with a transmembrane ligand of the Delta or Serrate/Jagged family on a neighbouring cell. Notch receptors regulate cell-fate choice in the development of many cell lineages, and so are vital in the regulation of embryonic differentiation due to development.

p53

A transcription factor that is activated by many genotoxic insults and that induces cellular senescence or apoptosis. The gene that encodes p53 is frequently mutated or functionally inactivated in cancer cells.

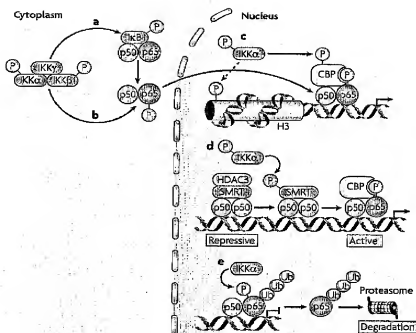


Figure 3 | New functions for IKK proteins in NF- κ B signalling. a, b | In addition to phosphorylating I κ B (inhibitor of nuclear factor- κ B (NF- κ B)) family members, I κ B kinases (IKKs) regulate transcriptional responses by phosphorylating NF- κ B proteins. c | IKK α is also found in the nucleus, may further promote transcription by inducing the phosphorylation of histone H3, which leads to greater κ B-site accessibility. IKK α also targets both transcriptional co-activators and co-repressors directly. Phosphorylation of the key co-activator CBP (CREB-binding protein) results in a binding preference of CBP for p55 over other transcription factors. d | IKK α also targets the SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) co-repressor complex, which associates with repressive NF- κ B complexes such as p50 homodimers, resulting in the displacement of histone deacetylase 3 (HDAC3) and the replacement of p50 homodimers with transcriptionally active NF- κ B complexes (for example, p50-p65 dimers). e | During late stages of the transcriptional response to pro-inflammatory stimuli, IKK α negatively regulates NF- κ B expression, presumably through phosphorylation and targeting of p55- and REL-containing (not shown) complexes for ubiquitination and degradation. Note that the green phosphate signifies phosphorylation that results in negative regulation of the phosphorylated protein, whereas the yellow phosphate indicates activation. Ub, ubiquitin.

we discuss the function of four I κ B proteins — BCL-3, I κ B α , I κ B β and p100 — with roles in NF- κ B signalling that have spurred this re-evaluation of the function of the I κ B family (FIG. 4).

BCL-3. The I κ B family member BCL-3 was originally identified as a proto-oncogene that was overexpressed in certain B-cell chronic lymphocytic leukaemias²². BCL-3 does not have a clearly defined role in NF- κ B signalling, although several unique properties of this protein have been described. BCL-3 functions as either a transactivating protein or as a traditional inhibitor of NF- κ B in a context-specific manner. The most striking characteristic of BCL-3, compared with other I κ B proteins, is that it has a well-defined TAD. BCL-3 is also mainly located in the nucleus (which is consistent with a role as a direct regulator of transcription)²³, where it forms complexes with p50 and p52 homodimers²⁴. These NF- κ B family members lack TADs and are therefore repressive, but binding to BCL-3 possibly confers transactivating

activity. Alternatively, BCL-3 might facilitate transcription by displacing the p50 and p52 homodimers and allowing transcriptionally active NF- κ B dimers to take their place. It is also possible that BCL-3 inhibits NF- κ B-dependent transcription by stabilizing p50 and p52 homodimers that are bound to κ B sites (FIG. 4b).

The ability of BCL-3 to facilitate or inhibit NF- κ B-dependent transcription is, in part, determined by post-translational modifications. BCL-3 is subject to several phosphorylation events that regulate its ability to bind to p50 or p52 homodimers and possibly confer transactivating activity^{25–29}. Therefore, the formation of transcriptionally active BCL-3-p50-p50 or BCL-3-p52-p52 complexes may require phosphorylation, as well as ubiquitination (see later), of BCL-3 (REFS 26, 27). However, the group of genes that is regulated by BCL-3 has yet to be described, with the exception of the NF- κ B-regulated cell proliferation gene cyclin D1. The expression of cyclin D1 is controlled by the binding of BCL-3-p52-p52 complexes to a κ B site in its promoter²⁸. However, when p53 is active the expression levels of BCL-3 decrease, and p52 homodimers bind to the same κ B site within the cyclin D1 promoter and repress transcription by recruiting HDAC1 (REF. 29).

In addition to phosphorylation, transcriptional activation by BCL-3 depends on its localization to the nucleus, which has recently been shown to depend on ubiquitination. BCL-3 ubiquitination is negatively regulated by the deubiquitinating enzyme cylindromatosis protein (CYLD), a negative regulator of NF- κ B signalling²⁷. Because CYLD is itself an NF- κ B-regulated gene, these results may help to clarify some of the confusion surrounding BCL-3 function: to form transcriptionally active complexes, BCL-3 must be induced and phosphorylated either in the absence of CYLD or under conditions in which BCL-3 resists deubiquitination by CYLD. When CYLD and BCL-3 are co-expressed, p50 or p52 homodimers are displaced from the promoter by deubiquitinated BCL-3 (REF. 27).

However, BCL-3 may also function as an inhibitor of NF- κ B activity by stabilizing repressive NF- κ B homodimers in a DNA-bound state and preventing the binding of transcriptionally active dimers. In fact, stabilization of repressive complexes through the induction of BCL-3 expression has been proposed to function in processes such as LPS tolerance³⁰. BCL-3 may prevent ubiquitination of p50 homodimers and their replacement with transcriptionally active NF- κ B dimers²⁵. Inhibition of NF- κ B by this mechanism would specifically occur at κ B sites that exhibit a strong binding preference for p50 homodimers, which may explain the existence of both LPS-tolerizable and LPS-non-tolerizable genes, as described recently³. Treatment of macrophages with the anti-inflammatory cytokine IL-10 induces the expression of BCL-3, and this leads to the inhibition of LPS-induced TNF production³¹. In addition, BCL-3 that is associated with p50 homodimers can regulate the expression of IL-10 in response to LPS³². BCL-3-p50-p50 complexes also negatively regulate the expression of the pro-inflammatory cytokine IL-23; IL-10-deficient mice produce increased levels of IL-23 owing to decreased

REVIEWS

binding of the BCL-3-p50-p50 NF- κ B complexes to the *IL23p19* promoter, which allows p65-containing NF- κ B complexes to induce transcription of this gene¹³. Thus, BCL-3 restricts inflammation by both suppressing pro-inflammatory gene expression and by inducing the expression of anti-inflammatory cytokines.

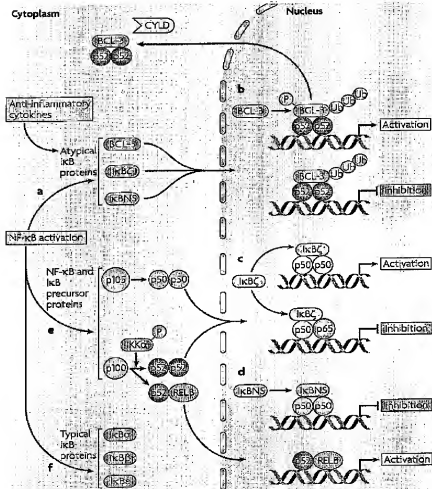


Figure 4 | New functions for I κ B family members. Nuclear factor- κ B (NF- κ B) activation results in the transcription of inhibitor of NF- κ B (I κ B) genes. a) *De novo* synthesis of the atypical I κ B proteins B-cell lymphoma 3 (BCL-3), I κ B ϵ , and I κ BNS results in the formation of complexes with transcriptionally incompetent p50 or p52 homodimers. b) BCL-3 can facilitate the transcription of a subset of NF- κ B-dependent genes by providing transactivating ability to the inhibitory p50 (not shown) or p52 homodimers when subjected to proper post-translational modifications [phosphorylation and ubiquitination] that are induced by pro-inflammatory signalling pathways. Non-phosphorylated but ubiquitinated BCL-3 that is induced by anti-inflammatory cytokines could also stabilize these inhibitory p50 or p52 homodimers that repress transcription. Alternatively, the deubiquitinating enzyme cylindromatosis protein (CYLD) can remove ubiquitin from BCL-3, which results in the sequestration of BCL-3–p52–p52 (or BCL-3–p50–p50) complexes in the cytoplasm. c) I κ B ϵ provides transcriptional ability to p50 homodimers when complexed with them. By contrast, when complexed with I κ BNS-containing dimers, I κ B ϵ can repress transcription. d) Interleukin-10 (IL-10) can repress transcription by stabilizing inhibitory p50 homodimers. e) Expression of the NF- κ B and I κ B precursor proteins p105 and p100 generates increasing pools of p50 and p52 homodimers and, in the case of p100, selectively stabilizes RELB-containing dimers. This provides both induced and constitutive NF- κ B activity that depends on I κ B kinase α (IKK α). f) Resynthesis of the typical I κ B proteins, especially I κ B α and I κ B β , provides negative feedback. Ub, ubiquitin.

I κ B α , *I κ B β* is more homologous to BCL-3 than to the other *I κ B* family members. *I κ B β* is not expressed constitutively, but is upregulated in response to specific NF- κ B signalling pathways, such as the myeloid differentiation primary response gene 88 (MyD88)-dependent signalling pathways that are activated by IL-1 and IL-17. Although other NF- κ B-inducing stimuli lead to *I κ B α* transcription, stabilization of the transcript and upregulation of *I κ B β* protein expression depends on additional signalling that is provided through Toll-like receptors (TLRs) or the IL-1 receptor (IL-1R) but not by TNF α . mRNA encoding *I κ B β* can also be stabilized by IL-17 stimulation, and co-stimulation with TNF and IL-17 can therefore induce levels of *I κ B β* that are similar to those that are induced in response to LPS or IL-1 (REF 34). It is unclear what shared signal between the IL-17 and IL-1R mediates this effect. However, rather than providing a negative feedback effect, such as that provided by typical *I κ B* proteins, *I κ B β* can positively regulate certain TNF- κ B dependent genes in response to TLR4 or IL-1R stimulation³⁵.

Similar to BCL-3, I κ B α associates with NF- κ B dimers that lack a TAD, especially p50 homodimers. Newly synthesized I κ B α associates with p50 homodimers that are bound to κ sites, for example, on the *h6* promoter. Consistent with this, there is no induction of IL-6 expression in I κ B α -deficient cells¹⁵. Unlike BCL-3, however, I κ B α does not have a well-defined TAD, although overexpression experiments suggest that it may have transactivating activity when bound to p50 (REF. 36). To complicate matters further, I κ B α may also negatively regulate p65-containing NF- κ B complexes, because it is capable of transactivation when bound to p50 but not when bound to p65 (REF. 39). Consistent with this differential role, there is a slight increase in the expression of some p65-dependent gene transcripts in I κ B α -deficient mice¹⁵. Therefore, I κ B α , similarly to BCL-3, can selectively inhibit or activate certain NF- κ B dimers (Fig. 4c). It should also be noted that in both cases there is probably also competition for promoter-bound NF- κ B dimers between typical I κ B family members and I κ B α or BCL-3.

IcBNS. IcBNS was first identified as an IκB family member that is inducibly expressed in T cells undergoing negative selection²⁹. Overexpression of IcBNS only partially inhibits NF-κB reporter-gene activity, and IcBNS has a preference for binding p50 *in vitro*³⁰. Subsequent work has shown that IcBNS expression can be induced in multiple tissues and, similarly to BCL-3 expression, is strongly induced by the anti-inflammatory cytokine IL-10 (REF 38). When IcBNS expression is reduced using RNA interference (RNAi), IL-10 fails to inhibit the induction of IL-6 after subsequent LPS stimulation³⁹. Thus, it was proposed that following IL-10 treatment of macrophages, induced IcBNS suppresses IL-6 production by associating with DNA-bound p50 homodimers and preventing the binding of transcriptionally active NF-κB dimers to DNA³⁸ (Fig. 4d).

In the absence of IκBNS, there is a selective increase in the production of LPS-induced IL-6 and IL-12p40 but not, for example, in the transcription of *Tnf*²⁹. Furthermore, IκBNS selectively regulates only the late

stages of IL-6 transcription in LPS-stimulated cells. In the absence of I κ BNS, the increased binding of p65 dimers to the *Il6* promoter was prolonged beyond the 3 hours observed in wild-type cells³⁹. Therefore, induced I κ BNS has a selective repressive effect on certain κ B sites in the promoters of pro-inflammatory target genes. Recently, it has also been shown that both BCL-3 and I κ BNS are induced following stimulation with LPS in regulatory DCs, a subset of DCs that produces high levels of IL-10 in response to LPS stimulation⁴⁰. Surprisingly, despite the initial observation that I κ BNS is inducibly expressed during thymocyte selection, I κ BNS-deficient mice have normal thymic development and T-cell function^{38,41}. There is a mild proliferative defect in I κ BNS-deficient T cells owing to decreased IL-2 production, which suggests that I κ BNS might increase *Il2* transcription⁴¹. Therefore, although I κ BNS bound to p50 homodimers on DNA is an inducible negative regulator of NF- κ B activity, it might also increase the expression of a small subset of genes. The mechanism by which it acts is yet to be defined.

p100. The precursor protein p100 adds another level of complexity to the I κ B family (FIG. 4e). Processing of p100 to generate the NF- κ B family member p52 occurs constitutively at a low level, but is also induced in response to certain NF- κ B stimuli that activate IKK α (REFS 42,43). p100 associates with several NF- κ B family members, but its main partner is RELB, which associates primarily with p100 or p52 and requires p100 binding for stability^{44,45}. As a result of selective binding of p100 to RELB, constitutive IKK α -dependent processing of p100 in certain tissues can significantly affect basal NF- κ B activity⁴⁶. Consequently, some tissues such as the thymus and the spleen, which contain increased numbers of RELB–p52 heterodimers, have decreased NF- κ B activity in RELB-deficient mice⁴⁷. Induction of p100 expression by NF- κ B can also facilitate the exchange of p65-containing heterodimers for heterodimers of RELB and p52 (REF. 48). In this context, replacing p65-containing heterodimers through the induction of RELB–p52 heterodimer formation could promote continued transcription by overcoming negative feedback mechanisms that target p65-containing complexes at late stages of the response: for example, at the *Ccl19* (CC-chemokine ligand 19) and *Ccl22* promoters⁴⁸. However, at other promoters, including those of *Il1b* and *Thf1*, RELB might act as a transcriptional repressor and might therefore maintain LPS tolerance owing to its induction at later time points^{48–50}.

Although stabilization of I κ B dimers may be regulated exclusively by p100, p100 itself can act more broadly in inhibiting NF- κ B dimers. p100 can regulate a fraction of canonical, p65-containing NF- κ B dimers through pathways that signal through IKK α (REF. 51). An increase in p100 and, consequently, p52 levels would promote the formation of RELB–p52 complexes, whereas increased p100 binding to canonical NF- κ B complexes would change the regulation of these complexes. For example, p100 is upregulated in T cells following T-cell receptor stimulation and forms complexes with p65-containing dimers⁵². This places canonical complexes under the

control of alternative-pathway stimuli that lead to the activation of IKK α and the degradation of p100. p100 therefore exhibits properties that are representative of several important I κ B functions. p100 is required for the stabilization of RELB, which indicates that I κ B proteins can help to determine the composition of NF- κ B dimers and thereby shape the transcriptional response.

Regulation of NF- κ B transcriptional activity

Specificity in transcriptional responses can, in part, be provided through the combinatorial effects of transcription factors. For NF- κ B, the complement of heterodimers and homodimers of the five family members that are selectively regulated by I κ B proteins, as previously discussed, is a primary source of transcriptional specificity⁷. However, there are additional factors that determine what constitutes an NF- κ B-regulated gene for a given stimulus (see Supplementary information S1 (box)). These factors can be necessary for the expression of many NF- κ B-regulated genes, as has recently been reported for the Akirin proteins, the mechanism of action of which is unknown. In addition, these factors can allow a given NF- κ B dimer to induce distinct transcriptional programmes in response to different stimuli. For example, phosphorylation of p65 at serine 276, which can be targeted by several kinases in diverse signalling pathways, is required for the induction of most, but not all, p65-regulated transcriptional responses⁵³. Cross-talk with other signalling pathways that are induced by many of the same stimuli as NF- κ B, such as the p38 pathway, shapes NF- κ B responses by regulating the activation of NF- κ B and by inducing changes in chromatin structure that are necessary for the activation of certain genes by NF- κ B. We hope that identifying these factors that are selectively required only for the transcription of certain NF- κ B target genes will provide a way of targeting inflammatory disease without crippling host immunity.

Akirin. The newly described protein Akirin is highly conserved throughout evolution and is required for the expression of a subset of NF- κ B-dependent genes (FIG. 5). There are two mammalian Akirin genes, *Akirin1* and *Akirin2*. Mice that lack *Akirin1* are overtly normal, and cells from these mice show a mild increase in IL-6 production in response to LPS, IL-1 β or MALP2 (macrophage-activating lipopeptide 2; a TLR2 and TLR6 agonist)⁵⁴. However, deletion of *Akirin2* leads to early embryonic lethality, and IL-6 production in *Akirin2*^{-/-} mouse embryonic fibroblasts is markedly decreased in response to several NF- κ B stimuli⁵⁴. Although degradation and resynthesis of I κ B α is relatively normal, the expression of genes such as BCL-3, CXC-chemokine ligand 10 (CXCL10) and CCL5 is also greatly reduced in the absence of Akirin2 (REF. 54). Akirin is a nuclear protein, and studies in *Drosophila melanogaster* suggest that Akirin might modify chromatin in certain NF- κ B-responsive genes, thereby allowing more efficient recruitment of NF- κ B to DNA. Consistent with a more general role in chromatin modification, loss of Akirin expression in *D. melanogaster* results in a more severe phenotype than loss of the NF- κ B homologue *Relish*⁵⁴.

RNA interference

The use of double-stranded RNAs (dsRNAs) with sequences that precisely match a given gene in order to knock down the expression of that gene by directing RNA-degrading enzymes to destroy the encoded mRNA transcript. The two most common forms of dsRNAs that are used for gene silencing are small—usually 21–base-pairs long—interfering RNAs and plasmid-delivered short hairpin RNAs.

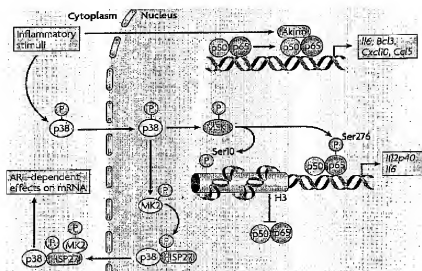


Figure 5 | Selective activation of NF- κ B transcriptional activity. The newly described nuclear protein Akt1 δ ins is required for the transcription of a subset of nuclear factor- κ B (NF- κ B)-dependent genes. Their mechanism of action is unclear, although it seems to be independent of nuclear localization and binding to κ B sites. The mitogen-activated protein kinase (MAPK) p38 governs an autoregulatory loop that affects a subset of genes that are transcribed by NF- κ B in response to inflammatory stimuli. Through mitogen- and stress-activated kinase 1 (MSK1), or potentially other histone kinases, p38 induces the phosphorylation of histone H3 at serine 10 and increases DNA accessibility for NF- κ B binding at specific promoters, for example, those of interleukin-12 p40 (IL12p40) and IL6. Activation of MSK1 may also directly increase transcription through the phosphorylation of p65 at serine 276. The p38 substrate MAPK-activated protein kinase-2 (MK2) phosphorylates heat-shock protein 27 (HSP27), which leads to the translocation of the HSP27-MK2 complex out of the nucleus. Cytoplasmic p38 further augments pro-inflammatory responses by increasing the translation of mRNA AU-rich element (ARE)-containing cytokines, including tumour-necrosis factor (TNF).

p38: histone modification at specific target genes.

Activation of NF- κ B during inflammation is accompanied by phosphorylation of histone H3 on specific target genes, and this can be mediated by the mitogen-activated protein kinase (MAPK) pathway. The use of specific inhibitors for the MAPK p38 (also known as MAPK14) in conjunction with LPS stimulation results in decreased phosphorylation of histone H3 at the *I κ B* α and *I κ B* promoters (8, 5 $^{\circ}$). The decrease in histone H3 phosphorylation at these promoters following p38 blockade and LPS stimulation is accompanied by delayed p65 binding, which suggests that p38-dependent phosphorylation of histone H3 at serine 10 increases p65 binding to specific promoter sites⁸. Interestingly, inhibition of p38 affects neither global DNA binding by NF- κ B nor transcription from promoters at which the phosphorylation of serine 10 does not depend on p38 (for example, for promoters of *I κ B α* transcription) or at which serine 10 phosphorylation is not evident (for example, for promoters of *Tf* transcription)⁹. The strength of MAPK signalling may therefore determine the selective transcriptional profiles of NF- κ B pathways, even those that use the same complement of NF- κ B dimers. The histone kinase that acts downstream of p38 has yet to be identified in this specific scenario. One reported p38 target is

mitogen- and stress-activated kinase 1 (MSK1), which might regulate the transcription factors CREB (cyclic-AMP-response-element binding protein) and NF- κ B, and can directly phosphorylate p65 (REF. 57). Indeed, it has been shown that p38 activates CREB to regulate the expression of genes that are downstream of TLR4 (REF. 58). Cooperation between CREB and NF- κ B, in addition to a direct effect of p38 on DNA accessibility for NF- κ B binding at specific promoters, may therefore contribute to the regulation of key components of the inflammatory response in an inflammatory milieu, particularly by facilitating macrophage survival through the upregulation of anti-apoptotic target genes⁵⁹.

MK2. MAPK-activated protein kinase-2 (MK2), also known as MAPKAPK2) has recently been implicated in dampening the activity of NF- κ B by regulating p38 (REF. 59) (FIG. 5). Well-characterized substrates of MK2 are the family of small heat-shock proteins (HSPs)⁶⁰, a mouse model of lung inflammation, phosphorylation of HSP27 and the production of NF- κ B-dependent pro-inflammatory cytokines are both decreased in MK2-deficient mice⁶¹. Mechanistically it seems that MK2 is required for the maintenance of DNA-bound NF- κ B. Knockdown of the expression of either HSP27 or MK2 decreases p65 nuclear retention and DNA binding⁶². This defect in NF- κ B nuclear binding in the absence of MK2 is evident primarily during the late stages of the response. Overexpression of a mutant form of HSP27 that cannot be phosphorylated has the same effect, which indicates that MK2 increases late transcriptional responses through this substrate⁶³. Interestingly, phosphorylation of p65 at serine 276 is increased at earlier time points in MK2-deficient cells and in cells that lack MK2 or HSP27 expression. This increase correlates with increased *I κ B α* transcription at early time points and decreased nuclear p65 levels at later time points⁶⁴.

There are several kinases that could be involved in the increased phosphorylation of p53 that is observed in the absence of MK2. Studies using RNAi-mediated knockdown suggest that a probable candidate for this is MSK1 (REF 58). As discussed earlier, MSK1, a known p38 kinase, can be regulated by p38 in many pathways that also activate NF- κ B. Furthermore, HSP27 or MK2 deficiency decreases the nuclear accumulation and total expression levels of p38 (REFS 61,62). Taken together, these data suggest the following mechanism (Fig. 5): MK2 promotes p38 exit from the nucleus by phosphorylating HSP27, thereby limiting MSK1 activation by p38 during the early stages of the NF- κ B response. However, MK2 also limits NF- κ B-induced negative feedback mechanisms, including IKBa transcription. MK2 and p38 probably also can function together in regulating stability of cytokine mRNA. Binding of NF- κ B and transcription from the *Thf* promoter does not depend on phosphorylation of histone H3 at serine 10 (REF 55). Instead, activation of p38 is, in part, responsible for the stabilization of *Thf* mRNA, which is crucial for the enhanced expression of TNF α . Therefore, expression of TNF protein, which is decreased in MK2 deficient cells, relies on the p38-MK2 pathway that promotes translation by acting on the *Thf* mRNA AU-rich element⁶⁴.

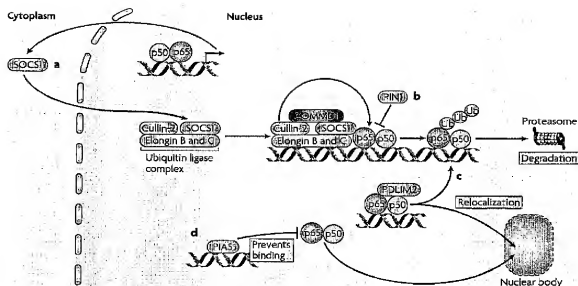


Figure 6 | Terminating NF- κ B transcriptional activity. a | Activation of nuclear factor- κ B (NF- κ B) induces the expression of suppressor of cytokine signalling 1 (SOCS1). In addition to targeting upstream signalling components (not shown), SOCS1 enters the nucleus, where it participates in the formation of the ECS (Elongin B–Elongin C–cullin-2–SOCS1) ubiquitin ligase complex. Signalling to NF- κ B also promotes the association of COMMD1 (copper metabolism Murr1) domain containing 1 with the ECS complex through binding of COMMD1 to SOCS1, and cullin-2. COMMD1 promotes binding of the ECS complex to p65-containing dimers and induces the ubiquitination and proteasomal degradation of these dimers. b | The prolyl isomerase PIN1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) modifies p65 at the SOCS1-binding site, which prevents the termination of NF- κ B activity by the ECS complex. c | PDLM2 (PDZ and LIM domain protein 2) interacts directly with p65 and promotes its relocalization to insoluble nuclear bodies and its ubiquitination and degradation. d | The small ubiquitin-like modifier (SUMO) ligase activity of PIAS (protein inhibitor of activated STAT) prevents binding of NF- κ B to certain promoters and may also mediate the localization of NF- κ B–DNA complexes to nuclear bodies, either for silencing or degradation. Ub, ubiquitin.

Terminating transcription and removing NF- κ B

In general, our understanding of the mechanisms that underlie the termination of NF- κ B activity has been more rudimentary than our understanding of the regulatory mechanisms that lead to its activation. It is generally accepted that the termination of NF- κ B activity relies mainly on the resynthesis of I κ B proteins, a model that is supported by studies on I κ B-deficient mice. However, this model does not explain how NF- κ B dimers that are bound to κ B DNA sequences are physically removed. It is unclear whether the I κ B family members that terminate the response act directly on DNA-bound NF- κ B or through some other mechanism. Recent studies have identified several proteins that seem to have specific roles in shutting off active NF- κ B. The mechanisms used by these newly described regulators include termination of activity through altered cofactor binding, degradation of active NF- κ B and displacement of NF- κ B dimers from the DNA. The first of these mechanisms was discussed earlier when describing the regulation of the binding of p65-containing dimers to HAT and HDAC complexes. In the next section, we discuss the second and third downregulatory mechanisms, which involve the ECS (Elongin B–Elongin C–cullin-2–SOCS1) ubiquitin ligase complex, PDLM2 (PDZ and LIM domain protein 2) and PIAS (protein inhibitor of activated STAT) (Fig. 6).

NF- κ B degradation by the ECS ubiquitin ligase complex. It has been suggested that at late stages of activation, nuclear p65-containing heterodimers are removed through ubiquitination and proteasomal degradation⁴⁵. Subsequent analyses suggested that this process might be mediated by IKK α -induced phosphorylation of p65 (and perhaps also REL), as discussed previously¹². However, the ubiquitin ligase (or ligases) that is responsible for nuclear NF- κ B degradation is unknown.

Suppressor of cytokine signalling-1 (SOCS1) participates in the formation of the ECS ubiquitin ligase complex and thereby decreases NF- κ B transcriptional activity^{46–48}. Independently of its role in regulating cytoplasmic signalling pathways, overexpression of SOCS1 decreases p65 stability⁴⁹. Recently the COMMD1 (copper metabolism Murr1) domain containing 1 protein, which inhibits NF- κ B and suppresses HIV replication, was also shown to associate with the ECS complex^{50,51}. Functionally, it seems that COMMD1 promotes associations between p65 and SOCS1 and between SOCS1 and cullin-2, which facilitates the ubiquitination of p65 by the ECS complex and its subsequent proteasomal degradation (Fig. 6). Although the expression levels of nuclear p65 and its target transcripts are increased following TNF stimulation in COMMD1-deficient cells, the kinetics of the termination of the transcriptional response are similar to those that are observed in control cells⁵². The accumulation of p65 in the nucleus was not sustained

in COMMD1-deficient cells owing to an increase in the resynthesis of I κ B³¹. Blocking *de novo* synthesis of proteins (including I κ B α) in COMMD1-deficient cells resulted in sustained high levels of nuclear p65 at late time points, which suggests that higher levels of I κ B α expression (owing to COMMD1 deficiency) during early stages of activation reduce the levels of nuclear p65 at late stages³¹. Therefore, COMMD1 targeting of a SOCS1-containing ubiquitin ligase complex to p65 is a significant contributor to the termination of the p65-mediated transcriptional response. However, even in the absence of COMMD1, nuclear p65 levels decrease at late time points relative to the peak of the response owing to I κ B α resynthesis, and not all NF- κ B-dependent genes exhibit a significant alteration in their expression profile. Therefore, other mechanisms in addition to ECS-mediated ubiquitylation and degradation of p65 have important roles in the termination of NF- κ B activity.

Under certain circumstances the ECS complex may be selectively regulated by PIN1 (peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1). It was initially observed that increased PIN1 expression in cancer cells correlated with induction of NF- κ B activity, and in overexpression experiments PIN1 could activate NF- κ B-dependent transcription independently of the IKK complex and degradation of I κ B. *Pin1*^{-/-} mouse embryonic fibroblasts are resistant to IL-1 stimulation and do not activate NF- κ B in response to TNF or LPS³². The PIN1-binding site in p65 overlaps with the SOCS1-binding site, and overexpression of PIN1 reverses the decrease in p65 stability that is observed following SOCS1 overexpression³³. However, a point mutant of p65 (threonine 254 to alanine) that disrupts PIN1 binding but not SOCS1 binding is highly unstable. Therefore, PIN1 seems to prevent ECS-induced ubiquitylation and degradation of nuclear p65-containing NF- κ B dimers.

NF- κ B degradation and silencing by PDLIM2. PDLIM2 is a nuclear ubiquitin ligase that has recently been shown to function in the negative regulation of p65-containing dimers³⁴ (FIG. 8), and PDLIM2-deficient mice exhibit uncontrolled inflammation³⁴. PDLIM2 has been suggested to function as both a p65 ubiquitin ligase and a targeting protein that translocates p65 to specific nuclear compartments known as PML nuclear bodies. Therefore, PDLIM2 can target both nuclear p65 for proteasomal degradation (analogous to the ECS complex) and also relocate DNA-bound p65 to areas of transcriptional silencing, thereby facilitating the inhibition of NF- κ B-dependent transcriptional activity. PDLIM2 also binds to actin and α -actinin, which are found in the nucleus and have been proposed to be involved in transcriptional regulation^{35,36}. Studies of mice that lack PDLIM2 expression suggest that it has a predominantly gene-silencing role³⁴, and that actin depolymerization enhances the transcription of NF- κ B-dependent cytokines, although this interaction has low specificity³⁷. At present, the mechanism by which the function of PDLIM2 is regulated is not known. It is possible that PDLIM2 is constitutively active and can act on p65 only after it enters the nucleus³⁷. Alternatively, although overexpression

of PDLIM2 can direct p65 to PML nuclear bodies, additional stimulus-induced signals could be required for the interaction of PDLIM2 with p65 under normal conditions. It is tempting to speculate that MK2 and HSP27, which regulate actin dynamics and exhibit subnuclear localization³⁸, also have a role in regulating the subnuclear localization of p65. Recently, it has been shown that p65 is crucial for the formation of the interferon- β (IFN β) enhancosome and for IFN β expression because it mediates the interchromosomal interaction of several regions with κ B binding sites³⁷.

PIAS1 inhibits p65 DNA binding. The PIAS family of proteins are small ubiquitin-like modifier (SUMO) E3 ligases that can inhibit signal transducer and activator of transcription (STAT) and NF- κ B transcriptional responses. Two members of this protein family, PIAS1 and PIASy (also known as PIAS4), have been shown to affect NF- κ B activity. PIAS1 was initially characterized as a regulator of STAT1. It was subsequently found that PIAS1-deficient mice exhibit increased expression levels of interferon-regulated pro-inflammatory cytokines and increased sensitivity to LPS-induced shock³⁹. These observations suggested that, in addition to interferon responses, there might be a link between PIAS1 and NF- κ B. Subsequent analyses of NF- κ B occupancy of κ B binding sites by chromatin immunoprecipitation and other *in vitro* analyses indicated that PIAS1 directly inhibits the DNA binding of p65-containing dimers³⁹ (FIG. 6). Intriguingly, the effect of PIAS1 on p65-induced transcription occurs at early time points. Therefore, PIAS1-deficient cells show significantly increased amounts of mRNA encoding I κ B α and IL-1 β 30 minutes or 1 hour after stimulation, but relatively normal levels at later time points³⁹.

Although loss of PIASy expression only mildly affects STAT1-mediated responses^{40,41}, PIASy-deficient mice, similarly to PIAS1-deficient mice, are hypersensitive to LPS-induced shock and exhibit increased cytokine induction⁴¹. Similarly to PIAS1-deficient cells, PIASy-deficient cells have increased DNA binding of p65-containing dimers, and this depends on the inducing stimulus⁴¹. However, the subset of NF- κ B-regulated genes that is affected by PIASy and PIAS1 is different. For example, loss of PIAS1 has a marked effect on TNF-induced I κ B α and IL-1 β expression, whereas loss of PIASy does not. By contrast, TNF-induced CXCL1 expression is affected by PIASy, but not by PIAS1 (REFS 78,81).

Despite their significant effects on the expression of certain NF- κ B-regulated genes, it is unclear how PIAS proteins are regulated and how they affect NF- κ B DNA-binding activity at immediate early target genes and only during the initial phase of the transcriptional response. For example, it is surprising that the expression of I κ B α or IL-1 β , which are NF- κ B target genes that are rapidly and robustly induced, is increased by the loss of PIAS1. Binding between NF- κ B and PIAS proteins does not seem to be regulated⁴¹. In fact, phosphorylation of PIAS1, which can occur in response to several stimuli, does not affect binding to overexpressed p65. However, transfection of PIAS1-deficient cells with a mutant PIAS1 that cannot be phosphorylated does not reconstitute PIAS1

PML nuclear body

A type of nuclear speckle of unknown function that contains several proteins, including the promyelocytic leukaemia (PML) protein.

Small ubiquitin-like modifier (SUMO) E3 ligase

An enzyme that catalyses the conjugation of SUMO to a protein substrate.

LPS-induced shock

A clinical condition that is induced by hyper-reactivity of the innate immune system to bacterial LPS. It is mediated by the pro-inflammatory cytokines interleukin-1 and tumour necrosis factor, both of which are produced in high amounts following sustained activation of Toll-like receptor 4 by LPS.

Box 1 NF- κ B in the resolution of inflammation

Although nuclear factor- κ B (NF- κ B) acts mainly as an initiator of inflammation, recent studies suggest that it also functions in the equally complex process of resolution of inflammation. Inhibition of NF- κ B after the initial inflammatory insult, and during the resolution phase, may in certain conditions prolong rather than inhibit inflammation, thereby delaying tissue repair⁴⁴. Ablation of IKK (inhibitor of NF- κ B) kinase) shows that this kinase has a role in the inflammatory response but, surprisingly, it is also necessary for tissue repair, including in conditions in which inflammation is the main mediator of tissue injury. This is evident in experiments in which conditional ablation of IKK β in enterocytes was induced in a model of ischaemia–reperfusion injury⁴⁵. IKK β deficiency in enterocytes was necessary for both the lethal systemic inflammatory response that results from gut ischaemia–reperfusion and for the repair of the damaged gut epithelium⁴⁵. Subsequent work has shown that ablation of IKK activity, through either deletion of the gene that encodes IKK γ or of the genes that encode IKK α and IKK β , is crucial for the maintenance of the colonic epithelium^{46,47}. These results are consistent with previous findings showing that activation of NF- κ B through Toll-like receptors in response to gut microorganisms or hyaluronic acid is necessary for the maintenance of the intestinal and lung epithelium^{48,49}. Therefore, although NF- κ B fulfils its expected role in inducing pro-inflammatory gene-expression programmes, it also surprisingly has an important role in the resolution of inflammation and in tissue repair.

Ischaemia–reperfusion injury

An injury in which the tissue first suffers from hypoxia as a result of severely decreased, or completely arrested, blood flow. Restoration of normal blood flow then triggers inflammation, which exacerbates tissue damage.

function⁴⁴. Furthermore, IKK α binds PIAS1 and mediates PIAS1 phosphorylation⁴⁵. The SUMO ligase activity of PIAS1 is required for the phosphorylation of PIAS1 and for the repression of NF- κ B- and STAT1-dependent transcriptional responses⁴⁵. Because the activation of the IKK complex in response to DNA damage has been suggested to involve PIASy⁴⁴, it is important to establish whether PIAS1 SUMO ligase activity is necessary for nuclear IKK α activation.

Concluding remarks

Orchestration of the inflammatory response is arguably the key physiological function of NF- κ B. The broad role of inflammation in pathological processes only serves to further highlight this aspect of the biology of NF- κ B. For this reason, there is considerable interest in the therapeutic inhibition of NF- κ B. However, several recent reports suggest that severe harmful consequences could result from the widespread inhibition of NF- κ B, including impaired resolution of inflammation (Box 1). In this Review, we discuss several new facets of NF- κ B regulation that may lead to the development of more selective approaches for NF- κ B inhibition. For example, preservation of IKK α activity may be important for terminating pro-inflammatory responses. The atypical IKK family members may facilitate the induction of antimicrobial responses while also being capable of dampening susceptibility to shock. As we have discussed for BCL-3, these functions are subject to regulation by post-translational modifications that may make them amenable to therapeutic intervention. Crosstalk with other important cellular pathways, such as p38 or p53, can alter the transcription of certain NF- κ B-dependent genes. Finally, the mechanisms that are also very important for the termination of NF- κ B activity are also very important for our understanding of the role of NF- κ B in normal physiology and disease. Research into these areas of NF- κ B biology will probably continue to generate significant new insights into the regulatory mechanisms of inflammation in the future.

- Hayden, M. S., West, A. F. & Chrest, S. NF- κ B and the immune response. *Cell* 71, 687–689 (2002).
- Foster, S. L., Hargreaves, D. C. & Reddy, A. H. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 447, 972–978 (2007).
- Sun, S. C. Deubiquitylation and regulation of the immune response. *Nature Rev. Immunol.* 8, 501–511 (2008).
- Schneider, C. I. κ B kinase complexes: gateways to NF- κ B activation and transcription. *Oncogene* 25, 6885–6705 (2006).
- Liu, Y. C., Penninger, J. & Karin, M. Immunity by ubiquitylation: a reversible process of modification. *Nature Rev. Immunol.* 5, 941–952 (2005).
- Chen, L. F. & Greene, W. C. Strapping the nuclear action of NF- κ B. *Nature Rev. Mol. Cell Biol.* 5, 352–401 (2004).
- Hoffmann, A., Natoli, G. & Chosh, G. Transcriptional regulation via the NF- κ B signaling module. *Oncogene* 25, 6706–6716 (2006).
- Havlen, M. S. & Chosh, S. Shared principles in NF- κ B signaling. *Cell* 132, 344–352 (2008).
- O'Neill, L. A. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity* 29, 12–20 (2008).
- Hacker, H. & Karin, M. Regulation and function of IKK and IKK-related kinases. *Cell* 172, 2006, 1–15 (2008).
- Zhang, H., Su, H., Erdemir, B., Romagne, H., Thompson, P. & Chosh, S. The transcriptional activity of NF- κ B is regulated by the phosphorylation of IKK α subunit through a cyclic AMP-independent mechanism. *Cell* 89, 415–424 (1997).
- Lavie, Y., T. Isakov, L. G. V. Nili, V. & Karin, M. IKK α limits macrophage NF- κ B activation and contributes to the resolution of inflammation. *Nature* 434, 1138–1143 (2005).
- Li, Q. et al. Enhanced NF- κ B activation and cellular function in macrophages lacking I κ B kinase 1 (IKK1). *Proc. Natl. Acad. Sci. USA* 102, 12425–12430 (2005). References 12 and 13 reveal the surprising role of IKK1 in limiting pro-inflammatory gene expression in macrophages.
- Anest, V. et al. A nucleosomal function for I κ B kinase-1 in NF- κ B-dependent gene expression. *Nature* 425, 639–643 (2003).
- Yamanoto, Y., Verma, U. N., Prasad, S., Kwek, Y. T. & Gaynor, R. B. Histone H3 phosphorylation by IKK α is critical for cytokine-induced gene repression. *Nature* 423, 655–659 (2003).
- Hoberg, J. E., Yang, F. & Mayo, M. W. SMIT denaturation by the I κ B kinase α is a prerequisite to NF- κ B transcription and survival. *Mol. Cell* 16, 245–255 (2004).
- Hoberg, J. E., Popko, A. E., Ramsey, C. S. & Mayo, M. W. I κ B kinase α -mediated repression of SMIT potentiates acetylation of RelA/p53 by p300. *Mol. Cell Biol.* 24, 437–471 (2004).
- Fernandes, M. J. et al. Nuclear IKK activity leads to dysregulated Notch-dependent gene expression in colorectal cancer. *Proc. Natl. Acad. Sci. USA* 104, 276–281 (2007).
- Huang, W. C., Ju, T. K., Hung, M. C. & Chen, C. Phosphorylation of CBP by IKK α promotes cell growth by switching the binding preference of CBP from p53 to NF- κ B. *Mol. Cell* 28, 75–87 (2007).
- Lu, J. L. et al. Nuclear cytokine-activated IKK α controls prostate cancer metastasis by repressing Maspin. *Nature* 446, 680–694 (2007).
- Zou, Z. et al. p53 regulates the expression of the lung suppressor gene maspin. *J. Biol. Chem.* 278, 6051–6054 (2003).
- Ohno, H., Takimoto, C. & McKeethen, T. W. The cancer suppressor gene *retinoblastoma* is related to genes implicated in cell lineage determination and cell cycle control. *Cell* 60, 991–997 (1990).
- Nolin, G. P. et al. The I κ B kinase complex contains a nuclear I κ B-like molecule that preferentially interacts with NF- κ B p50 and p52 in a phosphorylation-dependent manner. *Mol. Cell Biol.* 13, 3557–3566 (1993).
- Cammaro, J. H., Perez, P., Lira, S. A. & Bravo, R. Constitutive expression of Rel-1 in thymocytes increases the DNA binding of NF- κ B1/550 homodimers *in vivo*. *Mol. Cell Biol.* 16, 1542–1548 (1996).
- Bundy, D. L. & McKeethen, T. W. Diverse effects of BCL-3 phosphorylation on its modulation of NF- κ B p52 homodimer binding to DNA. *J. Biol. Chem.* 272, 33152–33159 (1997).
- Bour, V. et al. The oncoprotein Bcl-3 directly transactivates through κ B motifs via association with DNA-binding p50B homodimers. *Cell* 72, 729–739 (1993).
- This study shows that BCL-3 can induce the transcription of target genes by associating with NF- κ B p50 homodimers, which suggests that I κ B proteins could have complex roles in NF- κ B regulation.
- Masumori, R., Chmelarska, K., Hennrich, K., Pfeiffer, A. & Fasler, R. Cytokine-induced cell proliferation by blocking Bcl-3-dependent NF- κ B signaling. *Cell* 125, 555–577 (2006).
- Westorville, S. J., Mayo, M. W., Anest, V., Hanson, J. L. & Baldwin, A. S. Jr. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G₁ transition. *Mol. Cell Biol.* 21, 8428–8436 (2001).
- Rocha, S., Martin, A. M., Misk, D. W. & Perkins, N. D. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF- κ B subunit with histone deacetylase 1. *Mol. Cell Biol.* 23, 4713–4727 (2003).
- Wang, J. et al. BCL-3 and NF- κ B p50 attenuate lipopolysaccharide-induced inflammatory responses in macrophages. *J. Biol. Chem.* 279, 49995–50003 (2004).
- Cerny, R. J., Ruan, Q., Palmer, S., Hillard, R. & Chen, Y. H. Negative regulation of toll-like receptor signaling by NF- κ B p50 and p52 in the production of interleukin-6. *J. Biol. Chem.* 276, 673–678 (2001).
- Kawata, H. et al. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF- α production in macrophages. *J. Biol. Chem.* 276, 4125–4129 (2001).
- Mullbauer, M., Chilton, P. M., Mitchell, T. C. & Jobin, C. Impaired Bcl-3 up-regulation leads to enhanced IL-10-induced expression of interleukin-10 (IL-25p19) gene expression in IL-10⁺ mice. *J. Biol. Chem.* 275, 14162–14169 (2000).

Entrez Gene:

BCL-2 | ICBG | B

SUPPLEMENTARY INFORMATION
See online article S1 (box)

1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 2284, 2285, 2286, 2287, 2288, 2289, 2290, 2291, 2292, 2293, 2294, 2295, 2296, 2297, 2298, 2299, 2300, 2301, 2302, 2303, 2304, 2305, 2306, 2307, 2308, 2309, 2310, 2311, 2312, 2313, 2314, 2315, 2316, 2317, 2318, 2319, 2320, 2321, 2322, 2323, 2324, 2325, 2326, 2327, 2328, 2329, 2330, 2331, 2332, 2333, 2334, 2335, 2336, 2337, 2338, 2339, 2340, 2341, 2342, 2343, 2344, 2345, 2346, 2347, 2348, 2349, 2350, 2351, 2352, 2353, 2354, 2355, 2356, 2357, 2358, 2359, 2360, 2361, 2362, 2363, 2364, 2365, 2366, 2367, 2368, 2369, 2370, 2371, 2372, 2373, 2374, 2375, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2385, 2386, 2387, 2388, 2389, 2390, 2391, 2392, 2393, 2394, 2395, 2396, 2397, 2398, 2399, 2400, 2401, 2402, 2403, 2404, 2405, 2406, 2407, 2408, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2416, 2417, 2418, 2419, 2420, 2421, 2422, 2423, 2424, 2425, 2426, 2427, 2428, 2429, 2430, 2431, 2432, 2433, 2434, 2435, 2436, 2437, 2438, 2439, 2440, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2448, 2449, 2450, 2451, 2452, 2453, 2454, 2455, 2456, 2457, 2458, 2459, 2460, 2461, 2462, 2463, 2464, 2465, 2466, 2467, 2468, 2469, 2470, 2471, 2472, 2473, 2474, 2475, 2476, 2477, 2478, 2479, 2480, 2481, 2482, 2483, 2484, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2493, 2494, 2495, 2496, 2497, 2498, 2499, 2500, 2501, 2502, 2503, 2504, 2505, 2506, 2507, 2508, 2509, 2510, 2511, 2512, 2513, 2514, 2515, 2516, 2517, 2518, 2519, 2520, 2521, 2522, 2523, 2524, 2525, 2526, 2527, 2528, 2529, 2530, 2531, 2532, 2533, 2534, 2535, 2536, 2537, 2538, 2539, 2540, 2541, 2542, 2543, 2544, 2545, 2546, 2547, 2548, 2549, 2550, 2551, 2552, 2553, 2554, 2555, 2556, 2557, 2558, 2559, 2560, 2561, 2562, 2563, 2564, 2565, 2566, 2567, 2568, 2569, 2570, 2571, 2572, 2573, 2574, 2575, 2576, 2577, 2578, 2579, 2580, 2581, 2582, 2583, 2584, 2585, 2586, 2587, 2588, 2589, 2590, 2591, 2592, 2593, 2594, 2595, 2596, 2597, 2598, 2599, 2600, 2601, 2602, 2603, 2604, 2605, 2606, 2607, 2608, 2609, 2610, 2611, 2612, 2613, 2614, 2615, 2616, 2617, 2618, 2619, 2620, 2621, 2622, 2623, 2624, 2625, 2626, 2627, 2628, 2629, 2630, 2631, 2632, 2633, 2634, 2635, 2636, 2637, 2638, 2639, 2640, 2641, 2642, 2643, 2644, 2645, 2646, 2647, 2648, 2649, 2650, 2651, 2652, 2653, 2654, 2655, 2656, 2657, 2658, 2659, 2660, 2661, 2662, 2663, 2664, 2665, 2666, 2667, 2668, 2669, 2670, 2671, 2672, 2673, 2674, 2675, 2676, 2677, 2678, 26

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT J

Angiotensin II Stimulates the Release of Interleukin-6 and Interleukin-8 From Cultured Human Adipocytes by Activation of NF- κ B

Thomas Skurk, Vanessa van Harmelen, Hans Hauner

Objective—Several proinflammatory cytokines including IL-6 and IL-8 are produced by human adipocytes, but it is still unclear how this process is regulated. Angiotensin (Ang) II, which is also produced by adipocytes, might play a role as a regulator. In the present study, we investigated the effect of Ang II on the production of IL-6 and IL-8 in in vitro differentiated human adipocytes.

Methods and Results—Isolation of preadipocytes and differentiation of these cells into adipocytes, Real-time quantitative reverse-transcriptase polymerase chain reaction, Western-blot, enzyme-linked immunosorbent assay, and electrophoretic mobility shift assay. Ang II-stimulated IL-6 and IL-8 mRNA expression and protein release in a time- and concentration-dependent way. This action of Ang II was completely blocked by the NF- κ B-blocker Bay 117082 and the AT₁ blocker candesartan, but only partially by the AT₂-blocker PD 123 319. Incubation of adipocytes with Ang II resulted in an increased phosphorylation of the p65 subunit of NF- κ B and an increased translocation of NF- κ B to the nucleus.

Conclusion—Ang II stimulates IL-6 and IL-8 production and release from human adipocytes by a NF- κ B-dependent pathway. This proinflammatory action of Ang II seems to be mediated by the AT₁ and less by the AT₂ receptor subtype. (*Arterioscler Thromb Vasc Biol.* 2004;24:1199-1203.)

Key Words: cytokines ■ adipocytes ■ angiotensin ■ AT₁ receptor ■ NF- κ B

Human adipocytes are known to produce a variety of proteins, which are released into the circulation.¹ For instance, adipocytes secrete proinflammatory cytokines like interleukin (IL)-1 β , IL-6, and IL-8.²⁻⁴ Recently, plasma levels of IL-6 and other cytokines have been shown to be correlated with body mass index (BMI) and body fat mass;^{5,7} therefore, it has been suggested that these cytokines may represent the state of chronic inflammation observed in obesity. It is well-established that the expression and release of these cytokines in human adipocytes are subject to a complex hormonal regulation.^{3,8}

Adipocytes are also a major source of products of the renin-angiotensin-system (RAS), particularly of its active component angiotensin (Ang) II.⁹⁻¹⁰ It has recently been discovered that Ang II exerts transcriptional activity via an Ang II-responsive element in the IL-6 promoter in rodent cardiomyocytes.¹¹ Moreover, Ang II can activate the proinflammatory transcription factor NF- κ B in various cell types.¹²⁻¹⁴ Ang II has been shown to cause release of IL-6 from cultured vascular smooth muscle cells via a NF- κ B-dependent pathway.¹⁵ As described by Ruiz-Ortega et al.,¹² activation of NF- κ B may be attributed to both functional receptor subtypes AT₁ and AT₂. The possible modification of

the inflammatory process by Ang II may have clinical implications. Recent studies have shown a successful treatment of patients with atherosclerotic complications with angiotensin-converting enzyme (ACE) inhibitors and AT₁ receptor antagonists.¹⁶

The aim of the present study was to examine whether Ang II is involved in the release of proinflammatory cytokines in human adipocytes, particularly in the release of IL-6 and IL-8. In addition, we were interested to investigate whether a possible effect of Ang II on cytokine release is mediated by NF- κ B-dependent pathway. Moreover, we used selective Ang II receptor blockers to unravel the role of the AT₁ and AT₂ receptor subtypes in this context.

Methods

Subjects

Adipose tissue for the isolation of pre-adipocytes was obtained from healthy and normal-weight women (BMI ≤ 26 kg/m², age range 18 to 50 years) undergoing elective mammary reduction. All subjects were white and did not have acute infection, malignancies, or any other consuming disease. Informed consent was obtained from all subjects. The study was approved by the ethical committee of the Heinrich-Heine University in Düsseldorf.

Received March 23, 2004; accepted April 12, 2004.

From Else Kröner Fresenius Zentrum für Ernährungsmedizin der TU München, Freising-Weihenstephan, Germany.

T.S. and V.v.H. contributed equally to this article.

Correspondence to Dr. Thomas Skurk, Else Kröner Fresenius Zentrum für Ernährungsmedizin der TU München, Hochfeldweg 1, 85350 Freising-Weihenstephan, Germany. E-mail: skurk@wzz.tum.de

© 2004 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000131266.38312.2e

Pre-Adipocyte Differentiation

Adipocyte precursor cells were isolated and differentiated as described previously.¹⁷ To enhance differentiation, the adipogenic medium was supplemented with 1 μ M/ml troglitazone and 0.5 mmol/L isobutyl-methylxanthine (IBMX) for the first 3 days. All experiments were performed after 16 days in culture when at least 50% of the cells had developed the adipocyte phenotype.

Incubation of Adipocytes

The newly developed fat cells were incubated in the presence and absence of the following agents: Ang II (Sigma, Munich, Germany), candesartan (AT₁ receptor blocker; AstraZeneca, Wedel, PD 123 319; (AT₂ receptor blocker; Sigma, Munich), and Bay 117082 (NF- κ B blocker; Bionoll, Hamburg). The cells were pre-incubated for 1 hour with the respective blocker before Ang II was added. The blockers were not cytotoxic at the concentrations used as tested in an MTS test (data not shown).

RNA Isolation and Quantitative

Reverse-Transcription Polymerase Chain Reaction
Total RNA was isolated and reverse-transcribed as described previously.¹⁸ The specific mRNAs were determined by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using light-cycler technology (Roche Diagnostics).¹⁸ The primer sequences were as follows: IL-6 (accession: AF372214) 5'-TAG CCG CCC CAC ACA GAC AG and 3'-GGC TGG CAT TTG TGG TTG GG product length 407 bp, annealing temperature 57°C, melting temperature 84.5°C; IL-8 (accession: AF385628) 5'-AAC TTT GTA CCC CAC TTT GCT CT and 3'-AAC GGT TGC CTT TGT ATT TAT GGT; product length 479 bp, annealing temperature 65°C, melting temperature 85.6°C; Sp1 (accession: AF232284) 5'-GAG AGT GGC TCA CAG CCT GTC and 3'-GTT CAG AGC ATC AGA CCC CTC; product length 231 bp, annealing temperature 57°C, melting temperature 86.0°C. Sp1 was used as a reference gene.

Measurement of Proteins

For quantification of IL-6 and IL-8 protein in the culture medium, a commercially available enzyme-linked immunosorbent assay (ELISA) was used (HIS technologies, Freiburg, Germany). The intraassay and interassay coefficients of variation were $\leq 10\%$ and 5%, respectively.

Western Blot Analysis

Immunological detection of the phosphorylated form of the p65 subunit of NF- κ B was performed as described before.¹⁹

Electromobility Shift Assay and Supershift Assay

Nuclear extracts were prepared by lysing adipocytes in a buffer containing 20 mmol/L HEPES, pH 7.9, 10 mmol/L NaCl, 0.2 mol/L EDTA, 2 mmol/L DTT, and Complete (Roche Diagnostics GmbH, Mannheim, Germany). The cells were centrifuged and the pellet was resuspended in a buffer containing 2 mmol/L HEPES, pH 7.9, 0.75 mmol/L spermidine, 0.15 mmol/L spermin, 420 mmol/L NaCl, 0.2 mol/L EDTA, 2 mmol/L DTT, 25% glycerol, and Complete (Roche Diagnostics GmbH). Nuclear fragments were incubated for 30 minutes on ice and centrifuged; after that, the supernatant was used for the EMSA or the supershift assay. The NF- κ B probe had the following consensus sequence: 5'-AGT TGA GGG GAC TTT CCC AGG C (top strand only). The labeling of the probe and the binding reaction were performed using the DIG Gel shift kit (Roche Diagnostics GmbH). Samples were separated in a nondenaturing 6% PAGE gel, and bands were detected using a Lumi-imager device (Roche Diagnostics GmbH). For the supershift assay, antibodies specific for the subunits of NF- κ B: p70, p65, and p50, respectively, were included in the NF- κ B-binding reaction in a dilution of 1:10⁴.

Statistical Analysis

Results are expressed as mean \pm SD. A paired *t* test was used for statistical comparisons.

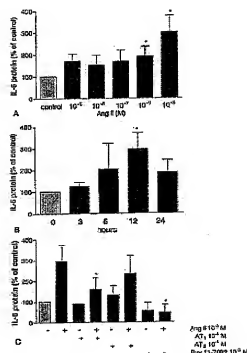


Figure 1. Concentration-dependent (A) and time-dependent (B) effects of Ang II on IL-6 protein release into the culture medium from in vitro differentiated human adipocytes. Ang II was added on day 16 after cells had acquired the adipocyte phenotype for the time periods and concentrations indicated. The incubation time was 24 hours (A), the Ang II concentration was 10⁻⁸ M (B). C, Cells were preincubated for 1 hour with or without the blockers indicated, followed by an incubation with Ang II with or without the blockers for 24 hours. Results represent mean \pm SD of 4 independent experiments in duplicate. A, B, **P* < 0.05 versus controls. C, **P* < 0.05 versus Ang II-stimulated cells.

Results

Effect of Ang II on IL-6 and IL-8 Release From Human Adipose Cells

Incubation of the cells was performed on day 16 when at least 50% of the stromal cells had acquired adipocyte morphology. Cells were incubated for 24 hours with increasing concentrations of Ang II. Under these conditions, there was a concentration-dependent increase in IL-6 and IL-8 release into the culture medium. The concentrations at which Ang II significantly enhanced IL-6 and IL-8 release were 10⁻⁸ M and 10⁻⁷ M, respectively (Figures 1A and 2A). At 10⁻⁸ M Ang II, the release of both IL-6 and IL-8 protein was increased 3- and 4-fold, respectively. In addition, there was a time-dependent increase in IL-6 and IL-8 release on exposure to 10⁻⁸ M Ang II. The stimulatory effect was observed after an incubation of 6 hours and remained stable for the whole incubation period (Figures 1B and 2B).

Effect of Ang II on mRNA Levels of IL-6 and IL-8

To address the question whether the stimulatory effect of Ang II is associated with changes at the transcriptional level, we measured mRNA levels of IL-6 and IL-8 in the absence and presence of Ang II. The peptide induced a time- and concentration-dependent increase in IL-6 and IL-8 mRNA compared with control cultures. The stimulatory effect was

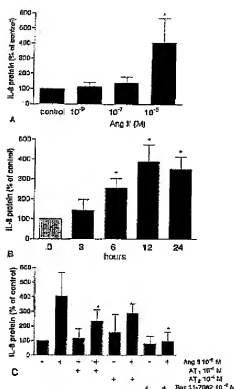


Figure 2. Concentration-dependent (A) and time-dependent (B) effects of Ang II on IL-8 protein release into the culture medium of in vitro differentiated human adipocytes. See Figure 1 for more information.

maximal after 6 hours of incubation (corresponding to a 2- to 3-fold increase of IL-6 and IL-8, respectively) ($P < 0.05$) (Table 1). The minimum stimulatory concentration of Ang II was 10^{-7} M and the maximum stimulatory concentration was 10^{-6} M (Table 1).

Effect of Candesartan, PD 123 319, and Bay 117082 on IL-6 and IL-8 Protein Release

To investigate whether the AT_1 or the AT_2 receptor subtype is involved in the effect of Ang II on IL-6 and IL-8 release, adipocytes were incubated with 10^{-6} M Ang II in the absence and presence of the specific AT_1 receptor blocker candesartan or the specific AT_2 receptor blocker PD 123 319. Candesartan significantly counteracted the Ang II-stimulated IL-6 and IL-8 release, although this inhibition was not complete (paired t test, Ang II versus Ang II plus candesartan, $P < 0.05$) (Figures 1C and 2C). PD 123 319 showed a tendency to inhibit Ang II-stimulated IL-6 and IL-8 release, but this effect did not reach statistical significance (Figures 1C and 2C).

To test whether Ang II induced IL-6 and IL-8 production via a signaling pathway involving NF- κ B, adipocytes were incubated with 10^{-6} M Ang II in the absence and presence of the specific NF- κ B blocker Bay 117082 (10^{-6} M). Bay 117082 prevented the stimulation of IL-6 and IL-8 release on Ang II exposure (paired t test Ang II versus Ang II plus Bay 117082; $P < 0.05$) (Figures 1C and 2C).

Effect of Ang II on NF- κ B Activation and Translocation

We next investigated whether Ang II is able to stimulate NF- κ B phosphorylation in adipocytes by using a Western blot analysis.

TABLE 1. Effect of Given Concentrations of Ang II on IL-6 and IL-8 mRNA Levels in In Vitro Differentiated Human Adipocytes for the Time Periods Indicated

Concentration Ang II (M)	Time (h)	IL-6 mRNA	IL-8 mRNA
0	24	100	100
10 ⁻⁸	24	108 \pm 18	207 \pm 48
10 ⁻⁷	24	203 \pm 37	181 \pm 118
10 ⁻⁶	24	232 \pm 57	520 \pm 153
0	0	100	100
10 ⁻⁵	3	112 \pm 27	127 \pm 25
10 ⁻⁶	6	254 \pm 65	156 \pm 86
10 ⁻⁶	12	147 \pm 68	299 \pm 56
10 ⁻⁶	24	143 \pm 25	188 \pm 46

Results are given as mean \pm SD from 3 to 5 independent experiments.

Cells were incubated with Ang II (10^{-6} M) for 0, 1, 2, 5, 10, 30, and 60 minutes, respectively. After an incubation of 5 minutes, a significant 2-fold increase of phosphorylation of the p65 subunit of NF- κ B was found ($P < 0.05$) (Figure 3).

To confirm the action of Ang II on NF- κ B translocation, an EMSA was performed on nuclear extracts from cells treated with or without 10^{-5} mol/L Ang II for 3 hours. It was clearly observed that Ang II increases NF- κ B translocation to the nucleus in adipocytes (Figure 4). Additionally, to confirm that the observed bands in the EMSA were specific for NF- κ B, we performed a supershift assay that included NF- κ B subunit antibodies in the binding reaction of Ang II-treated nuclear extracts. A clear reduction in the intensity of the original NF- κ B probe complex was observed with the p50 and p65 antibody, but not with the p70 antibody (Figure 1, available online at <http://atvb.ahajournals.org>). Therefore, the observed band in the EMSA seemed to be specific for the p50/p65 heterodimer.

Ang II Stimulates NF- κ B Translocation via AT_1 Receptors

To study whether the effect of Ang II on NF- κ B is mediated via the AT_1 and/or the AT_2 receptor, the effect of candesartan and PD 123 319 on Ang II-stimulated NF- κ B translocation was investigated. Candesartan abolished Ang II-induced NF- κ B translocation significantly (Figure 4, $P < 0.05$), whereas Bay 117082 showed a tendency to reduce the stimulatory effect of Ang II on NF- κ B activation.

The Effect of Repetitive Addition of Ang II on IL-6 Release in Adipocytes

In most of the experiments, Ang II was used at a supraphysiological concentration of 10^{-5} M. The reason for using this concentration was that Ang II is rapidly degraded under culture conditions.²⁰ To show that Ang II also stimulates IL-6 secretion at lower concentrations, we performed additional experiments in which we added Ang II at a concentration of 10^{-9} M every 6 hours for 24 hours. The stimulation of IL-6 protein release was similar as compared with 10^{-5} M Ang II administered once (Table 2). Moreover, Ang II at 10^{-9} M stimulated NF- κ B translocation to the nucleus in adipocytes (data not shown).

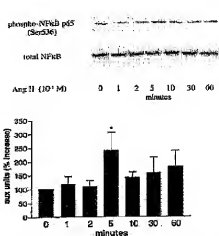


Figure 3. A representative Western blot of adipocytes treated with 10^{-8} M Ang II for different time intervals (upper panel). An antibody specific for phosphorylated p65 NF-κB was used. Blots were subsequently stripped and reprobed with an antibody recognizing both the phosphorylated and the unphosphorylated form of NF-κB. The columns in the lower panel show densitometric scanings and have means \pm SD of 4 experiments. Values are expressed as percent increase compared with control; 0=baseline without Ang II incubation, * $P < 0.05$.

Discussion

It is well-established that adipocytes release substantial amounts of the cytokines IL-6 and IL-8, and it is apparent that this release is subject to complex regulation.⁴ In the current study, we show for the first time to our knowledge that Ang II stimulates gene expression and protein release of IL-6 and IL-8 from human adipocytes in a time- and concentration-dependent fashion. The stimulation of IL-6 release by Ang II has been shown previously for other cell types in rodents.^{15,21} The production of IL-6 by adipocytes may be of special importance because up to one-third of circulating IL-6 is secreted by adipose tissue.²² In addition, the circulating levels of IL-8 are positively correlated with BMI, indirectly suggesting that adipose tissue may also release IL-8 into the blood.²³

It is known from recent studies that the effect of Ang II on inflammation involves an activation of the NF-κB signaling pathway.^{13,14,24} In the current study, we found that Ang II stimulates phosphorylation of the p65 subunit of NF-κB, using Western blot analysis. In addition, an EMSA revealed that Ang II induces translocation of NF-κB to the nucleus in human adipocytes. Further experiments demonstrated that blockade of NF-κB by Bay 117082 abolished the Ang II-mediated stimulation of IL-6 and IL-8 release. Taken together, these results strongly suggest that Ang II is mediating its effects on IL-6 and IL-8 via NF-κB activation.

In adipose tissue, most effects of Ang II are mediated via the AT₁ receptor, although there is evidence that the AT₂ receptor subtype is also expressed.²⁵ The receptor subtypes may share common signaling pathways leading to subsequent activation of NF-κB.²⁴ By using candesartan, a specific antagonist to the AT₁-receptor subtype, we found that the Ang II-mediated effects on NF-κB signaling and subsequent cytokine release were nearly completely abolished. When studying the effect of the AT₁ receptor antagonist PD 123 319 in our model, a small but not significant inhibition was

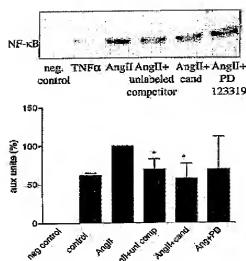


Figure 4. Electromobility shift assay showing Ang II-induced translocation of NF-κB. Cells were treated for 3 hours with 10^{-8} M Ang II after pretreatment with the indicated blockers for 1 hour. The negative control was a binding reaction without the NF-κB probe. Data are presented as percentage of the Ang II-stimulated samples, defined as 100%, of 4 experiments. cand indicates candesartan; PD, PD 123 319; unlabeled competitor, which is the same NF-κB-binding sequence without DIG-labeling. The effect of the blockers was analyzed using a paired *t* test. * $P < 0.05$ versus Ang II effect.

observed. These results indicate that signal transduction via the AT₁ receptor is the predominant pathway for Ang II-stimulated NF-κB activation and cytokine release in newly differentiated human adipocytes.

One has to keep in mind that degradation of Ang II released by human adipocytes in primary culture occurs very rapidly,²⁰ which makes it difficult to determine the true concentrations of Ang II required to stimulate cytokine production. Because of the high degradation rate of Ang II, we decided to use Ang II at a supraphysiological concentration of 10^{-5} M in most of our experiments. However, when differentiated adipocytes were incubated with Ang II at a physiological concentration of 10^{-8} M, and when the Ang II was added every 6 hours, the stimulation of IL-6 protein release was similar as compared with the supraphysiological concentration of Ang II (10^{-5} M) administered once. Moreover, Ang II at 10^{-8} M stimulated translocation of NF-κB in EMSA experiments. These results suggest that Ang II exerts its stimulating effects on cytokine production at near-physiological concentrations.

TABLE 2. Effect of Ang II on IL-6 Protein Secretion From In Vitro Differentiated Human Adipocytes

	Ang II	Ang II + Candesartan
In vitro differentiated adipocytes, Ang II (10^{-8} M), n=5	296 \pm 76	90 \pm 2
In vitro differentiated adipocytes, Ang II (10^{-8} M, every 6 h), n=3	226 \pm 20	78 \pm 7

Ang II was added either at a concentration of 10^{-8} M or at a concentration of 10^{-5} M repetitively every 6 hours. The AT₁ blocker candesartan was used at a concentration of 10^{-6} M. IL-6 protein was determined in the culture medium after 24 hours of incubation using a specific ELISA. Basal (nonstimulated) IL-6 protein concentration was defined as 100%. Results are given as mean \pm SD.

Obesity is considered as a state of subclinical chronic inflammation. This view is based on the finding that plasma levels of cytokines are elevated in obesity. This is most evident for IL-6, in which a positive association between circulating levels and BMI has been reported.^{6,7} At present, weight loss by dietary intervention or other methods have been found to be associated with decreased levels of IL-6 and other cytokines.^{26,27} It is rather unclear which factors are responsible for the upregulation of proinflammatory proteins in obesity. One possible mediator for this process might be Ang II. Angiotensinogen and all components of the renin-angiotensin system are produced by adipocytes⁸⁻¹⁰ and the expression of angiotensinogen is upregulated in obesity.^{28,29} Moreover, studies on the long-term use of ACE inhibitors and AT₁ blockers in patients with atherosclerosis and diabetes mellitus have suggested that these drugs have antiinflammatory properties.^{30,31} Therefore, in view of our and former results, a role of Ang II in the pathophysiology of the proinflammatory state is rather likely.

In conclusion, the results of this experimental study indicate that Ang II stimulates production and release of IL-6 and IL-8 in *in vitro* differentiated human adipocytes. This stimulatory action seems to be mediated via the AT₁ receptor and a NF- κ B-dependent signaling pathway.

References

- Ahima RS, Flier JS. Adipose tissue as an endocrine organ. *Trends Endocrinol Metab*. 2000;11:327-332.
- Zhang HH, Kumar S, Barnett AS, Eggo MC. Dexamethasone inhibits tumor necrosis factor- α -induced apoptosis and interleukin-1 β release in human subcutaneous adipocytes and preadipocytes. *J Clin Endocrinol Metab*. 2001;86:2817-2825.
- Braun JM, Pedersen SB, Richelsen B. Regulation of interleukin-8 production and gene expression in human adipose tissue *in vitro*. *J Clin Endocrinol Metab*. 2001;86:1267-1273.
- Pfith G, Bornstein SR, Gumbia M, Chrousos GP, Scherbaum WA, Hmmer H. Human breast adipocytes express interleukin-6 (IL-6) and its receptor system; increased IL-6 production by β -adrenergic activation and effects of IL-6 on adipocyte function. *J Clin Endocrinol Metab*. 2001;86:2281-2288.
- Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab*. 1998;83:847-850.
- Vgontzas AN, Papadimitriou DA, Bixler EO, Kales A, Tyson K, Chrousos GP. Elevation of plasma cytokines in disorders of excessive daytime sleepiness: role of sleep disturbance and obesity. *J Clin Endocrinol Metab*. 1997;82:3133-3136.
- Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab*. 2001;280:E745-E751.
- Karlsson C, Lindahl K, Ottosson M, Sjostrom L, Carlsson B, Carlsson LM. Human adipose tissue expresses angiotensinogen and enzymes required for its conversion to angiotensin II. *J Clin Endocrinol Metab*. 1998;83:3525-3529.
- Schling P, Mallow H, Trindl A, Löffler G. Evidence for a local renin-angiotensin system in primary cultured human preadipocytes. *Int J Obes Relat Metab Disord*. 1999;23:336-341.
- Engeli S, Gorzelitz K, Krautz R, Runkel N, Dietler A, Sharma AM. Co-expression of renin-angiotensin system genes in human adipose tissue. *J Hypertens*. 1999;17:555-560.
- Sano M, Fukuda K, Kodama H, Pan J, Saito M, Matsuzaki J, Takahashi T, Makino S, Kato T, Ogawa S. Interleukin-6 family of cytokines mediates angiotensin II-induced cardiac hypertrophy in rodent cardiomyocytes. *J Biol Chem*. 2000;275:29717-29723.
- Ruiz-Ortega M, Lorenzo O, Ruperez M, Konig S, Wittig B, Egidio J. Angiotensin II activates nuclear transcription factor kappaB through AT₁ and AT₂ in vascular smooth muscle cells: molecular mechanisms. *Circ Res*. 2000;86:1266-1272.
- Jamaluddin M, Meng T, Sun J, Boldogh I, Han Y, Brasier AR. Angiotensin II induces nuclear factor (NF)-kappaB1 isoforms to bind the angiotensinogen gene acute-phase response element: a stimulus-specific pathway for NF-kappaB activation. *Mol Endocrinol*. 2000;14:99-113.
- Brasier AR, Jamaluddin M, Han Y, Patterson C, Runge MS. Angiotensin II induces gene transcription through cell-type-dependent effects on the nuclear factor-kappaB (NF-kappaB) transcription factor. *Mol Cell Biochem*. 2000;212:155-169.
- Han Y, Runge MS, Brasier AR. Angiotensin II induces interleukin-6 transcription in vascular smooth muscle cells through pleiotropic activation of nuclear factor-kappa B transcription factors. *Circ Res*. 1999;84:695-703.
- Navalkar S, Parthasarathy S, Santanam N, Khan BV. Irbesartan, an angiotensin type 1 receptor inhibitor, regulates markers of inflammation in patients with premature atherosclerosis. *J Am Coll Cardiol*. 2001;37:440-444.
- Hauner H, Skurk T, Wabitsch M. Cultures of human adipose precursor cells. *Methods Mol Biol*. 2001;155:239-247.
- Skurk T, Lee YM, Hauner H. Angiotensin II and its metabolites stimulate PAI-1 protein release from human adipocytes in primary culture. *Hypertension*. 2001;37:1336-1340.
- van Harmelen V, Skurk T, Rohrig K, Hauner H. HMG-CoA reductase inhibitor cerivastatin inhibits interleukin-6 expression and secretion in human adipocytes. *Horm Metab Res*. 2003;35:466-470.
- Schling P, Schäfer T. Human adipose tissue cells keep tight control on the angiotensin II levels in their vicinity. *J Biol Chem*. 2002;277:48066-48075.
- Moriyama T, Fujibayashi M, Fujiwara Y, Kaneko T, Xia C, Imai E, Kamada T, Ando A, Ueda N. Angiotensin II stimulates interleukin-6 release from cultured mouse mesangial cells. *J Am Soc Nephrol*. 1995;6:95-101.
- Mokmed-Ah Y, Goodrick S, Rawesh A, Kaz DR, Miles JM, Yudkin JS, Klein S, Coppock SW. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor- α , *in vivo*. *J Clin Endocrinol Metab*. 1997;82:4196-4200.
- Straczekowski M, Dzienisz-Straczekowska S, Stepień A, Kowalska I, Szlachetkowska M, Kinalski L. Plasma interleukin-8 concentrations are increased in obese subjects and related to fat mass and tumor necrosis factor- α system. *J Clin Endocrinol Metab*. 2002;87:4602-4606.
- Ruiz-Ortega M, Lorenzo O, Suzuki Y, Ruperez M, Egidio J. Proinflammatory actions of angiotensins. *Curr Opin Nephrol Hypertens*. 2001;10:321-329.
- Crandall DL, Herzlinger HJ, Saunders BD, Armellino DC, Kral JG. Distribution of angiotensin II receptors in rat and human adipocytes. *J Lipid Res*. 1994;35:1378-1385.
- Zaccardi P, Nappo F, Guglielmo G, Esposito K, Marfella R, Cioffi M, D'Andrea F, Molinaro AM, Giugliano D. Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. *Circulation*. 2002;105:804-809.
- Gallist S, Sudi KM, Aigner R, Borkenstein M. Changes in serum interleukin-6 concentrations in obese children and adolescents during a weight reduction program. *Int J Obes Relat Metab Disord*. 2001;25:1640-1643.
- Van Harmelen V, Arias-Parr P, Hoffstedt J, Lundkvist I, Brinman S, Arner P. Increased adipose angiotensinogen gene expression in human obesity. *Obes Res*. 2000;8:337-341.
- Giacchetti G, Falao E, Sardu C, Camilioni MA, Marinello B, Gatti C, Garrapa GG, Guerrieri M, Mantero F. Gene expression of angiotensin in adipose tissue of obese patients. *Int J Obes Relat Metab Disord*. 2002;26:S142-S143.
- Scholkens BA, Landgraf W. ACE inhibition and atherogenesis. *Can J Physiol Pharmacol*. 2002;80:354-359.
- Lapointe N, Rouleau JL. Cardioprotective effects of vasopressinase inhibitors. *Can J Cardiol*. 2002;18:415-420.

**US PATENT APPLICATION
SERIAL NO. 10/721,892**

**DECLARATION OF
PROFESSOR THOMAS UNGER**

EXHIBIT K

Regulation of Inhibitory Protein- κ B and Monocyte Chemoattractant Protein-1 by Angiotensin II Type 2 Receptor-Activated Src Homology Protein Tyrosine Phosphatase-1 in Fetal Vascular Smooth Muscle Cells

LAN WU, MASARU IWAI, ZHEN LI, TETSUYA SHIUCHI, LI-JUAN MIN, TAI-XING CUI, JIAN-MEI LI, MIDORI OKUMURA, CLARA NAHMIA, AND MASATSUGU HORIUCHI

Department of Medical Biochemistry, Ehime University School of Medicine (L.W., M.I., Z.L., T.S., L.-J.M., T.-X.C., J.-M.L., M.O., M.H.), Shigenobu, Onsen-gun, Ehime 791-0295, Japan; and Centre National de la Recherche Scientifique, UPR0415-Institut Cochin de Genetique Molculaire (C.N.), Paris, France

In the present study we examined the effects of angiotensin II (Ang II) type 2 (AT_2) receptor stimulation on AT_1 receptor-mediated monocyte chemoattractant protein-1 (MCP-1) expression and the possible mechanisms of AT_2 receptor-mediated signaling in cultured rat fetal vascular smooth muscle cells, which express both AT_1 and AT_2 receptors. Ang II stimulation induced MCP-1 mRNA expression as well as an increase in nuclear factor- κ B (NF- κ B) binding to the corresponding *cis* DNA element of the MCP-1 promoter region and a decrease in the cytosolic inhibitory protein- κ B ($I\kappa$ B) protein level via AT_1 receptor stimulation, whereas stimulation of the AT_2 receptor decreased Ang II-induced MCP-1 expression, NF- κ B DNA binding, and $I\kappa$ B degradation, suggesting that activation of the AT_2 receptor attenuated AT_1 receptor-mediated MCP-1 expression via a decrease in NF- κ B DNA binding and an increase in $I\kappa$ B stability. Moreover, we demonstrated that AT_2 receptor

stimulation attenuated TNF α -mediated NF- κ B activation and MCP-1 expression. A tyrosine phosphatase inhibitor, orthovanadate, attenuated the AT_2 receptor-mediated increase in $I\kappa$ B protein. Moreover, we observed that two $I\kappa$ B subunits ($I\kappa$ B α and $I\kappa$ B β) were tyrosine-phosphorylated after Ang II stimulation. Transfection of a dominant-negative Src homology protein tyrosine phosphatase-1 mutant into vascular smooth muscle cells inhibited the AT_2 receptor-mediated increase in $I\kappa$ B, leading to a significant increase in AT_1 receptor-induced NF- κ B activation and MCP-1 expression. Taken together, our results demonstrated that AT_2 receptor stimulation attenuated MCP-1 expression via $I\kappa$ B stabilization, and Src homology protein tyrosine phosphatase-1 might play a critical role in the transcriptional regulation of MCP-1 expression through the control of $I\kappa$ B protein stability. (*Molecular Endocrinology* 18: 666-678, 2004)

ANGIOTENSIN (ANG) II is a potent mediator of oxidative stress and stimulates the release of cytokines and the expression of leukocyte adhesion molecules that mediate vessel wall inflammation. Ang II also acts as a direct growth factor for vascular smooth muscle cells (VSMC) and can stimulate the local production of metalloproteinases and plasminogen acti-

vator inhibitor. Most of the known physiological actions of Ang II are thought to be mediated via the Ang II type 1 (AT_1) receptor, but still less is known about the function of the Ang II type 2 (AT_2) receptor. Interestingly, the AT_2 receptor is up-regulated in certain pathological conditions, such as inflammation and vascular injury, suggesting that the AT_2 receptor plays an important role in vascular remodeling (1). It has been demonstrated that Ang II is a potent stimulator of monocyte chemoattractant protein-1 (MCP-1) expression in many cell types, including VSMC *in vivo* and *in vitro*, and that AT_1 receptor stimulation increases inflammation and stimulates atherosclerosis by stimulating MCP-1 expression (2, 3). We have recently demonstrated *in vivo* that MCP-1 expression in the injured artery was exaggerated in AT_2 receptor-null mice, suggesting that AT_2 receptor stimulation might inhibit MCP-1 expression (4). However, the molecular and cellular mechanisms of the regulation of MCP-1 ex-

Abbreviations: Ang II, angiotensin II; AT_1 , angiotensin II type 2; dn, dominant negative; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; $I\kappa$ B, inhibitory protein- κ B; IKK, inhibitory protein- κ B kinase; MCP-1, monocyte chemoattractant protein-1; MEK, MAPK kinase; NF- κ B, nuclear factor- κ B; NO, nitric oxide; PMSF, phenylmethylsulfonyl fluoride; SHP-1, Src homology protein tyrosine phosphatase-1; α -SM, α -smooth muscle; VSMC, vascular smooth muscle cell.

Molecular Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

pression by the AT₂ receptor are an enigma and need to be addressed.

Like many other proinflammatory genes, activation of the MCP-1 gene is controlled by the transcription factor, nuclear factor- κ B (NF- κ B). NF- κ B is present in the cytosol of unstimulated cells in an inactive form bound to the inhibitory protein- κ B (I κ B) (5). The ankyrin repeat domains of I κ B proteins prevent nuclear translocation of NF- κ B by masking the nuclear localization sequences of NF- κ B. In response to inflammatory stimuli, I κ B was phosphorylated and targeted for ubiquitination and degradation. Dissociation and degradation of I κ B result in activation of NF- κ B, which may be defined as translocation of the NF- κ B complex from the cytoplasm to the nucleus. After translocation to the nucleus, NF- κ B binds specific promoter elements of DNA and induces transcription of relevant genes, such as MCP-1.

The AT₂ receptor subtype is a member of the G protein-coupled receptor superfamily with relatively low sequence homology with the AT₁ receptor subtype. Accumulating evidence has revealed opposite intracellular effects of the AT₁ and AT₂ receptors, particularly on the regulation of protein kinases and phosphatases (6, 7). It is postulated that the final response to Ang II stimulation in cells coexpressing AT₁ and AT₂ receptors is dependent on the relative expression levels of these two receptors. The growth inhibitory effects of the AT₂ receptor have been shown to be associated with the activation and/or induction of a series of phosphatases (1), including the protein tyrosine phosphatase Src homology protein tyrosine phosphatase-1 (SHP-1) (8), MAPK phosphatase-1 (9), and serine/threonine phosphatase 2A (10), which results in the inactivation of AT₁ receptor- and/or growth factor-activated ERK. Therefore, we postulated that the AT₂ receptor-activated specific phosphatase(s) antagonizes the signaling pathway involved in AT₁ receptor-mediated MCP-1 production. To explore the roles of signaling cross-talk of the AT₁ and AT₂ receptors in the regulation of MCP-1 expression, we focused on NF- κ B/I κ B. In fetal VSMC, which endogenously express both AT₁ and AT₂ receptors.

RESULTS

Inhibition of MCP-1 Expression by AT₂ Receptor Stimulation

To examine the role of the endogenous AT₂ receptor in MCP-1 expression, we employed fetal VSMC prepared from the rat aorta on embryonic d 20, which express both AT₁ and AT₂ receptors. Receptor binding assay revealed that fetal VSMC expressed both AT₁ and AT₂ receptors (AT₁ receptor, 11.30 ± 0.35 fmol/ 10^6 cells; AT₂ receptor, 6.33 ± 1.01 fmol/ 10^6 cells; $n = 6$; mean \pm SE) as previously described (11). We did not observe any apparent changes in Ang II receptor subtype binding in the following experimental conditions.

Ang II increased MCP-1 mRNA expression assessed by Northern blot. Ang II-mediated MCP-1 expression peaked at 4 h and declined gradually thereafter (Fig. 1A). Stimulation with Ang II in the presence of an AT₂ receptor-specific antagonist, PD123319, further increased MCP-1 expression, whereas addition of an AT₁ receptor blocker, valsartan, decreased Ang II-mediated MCP-1 expression (Fig. 1B). We did not observe any significant difference in MCP-1 expression between control VSMC and VSMC treated with Ang II and valsartan. Neither valsartan nor PD123319 alone influenced MCP-1 expression. These results suggest that AT₂ receptor stimulation antagonized AT₁ receptor-mediated MCP-1 mRNA expression. To further clarify the possibility that AT₂ receptor stimulation inhibits MCP-1 expression, we examined the effect of CGP42112A, an AT₂ receptor agonist, on TNF α -induced MCP-1 expression, as it has been reported that TNF α stimulation enhances MCP-1 expression via enhancement of NF- κ B binding and inhibition of I κ B protein degradation in VSMC (12–14). We observed that CGP42112A decreased TNF α -induced MCP-1 expression (Fig. 1B).

Regulation of Nuclear NF- κ B Binding and Degradation of Cytosolic I κ B via Cross-Talk of AT₁ and AT₂ Receptors

To examine Ang II receptor subtype-regulated NF- κ B binding, we performed electrophoretic mobility shift assay (EMSA) using nuclear extracts prepared from fetal VSMC stimulated with Ang II for 30, 60, 90, and 120 min and a ³²P-labeled NF- κ B-binding oligonucleotide probe and observed a time-dependent increase in the complex formation (Fig. 2A) that reached a peak after 90 min of Ang II stimulation. As shown in Fig. 2B, addition of a 50-fold excess of unlabeled NF- κ B-binding oligonucleotide as a competitor abolished Ang II-induced NF- κ B binding, but the addition of a mutant NF- κ B-binding oligonucleotide did not influence this binding. Incubation of nuclear extract with anti-NF- κ B antibodies decreased this binding. Stimulation with Ang II in the presence of PD123319 further increased NF- κ B binding, whereas addition of valsartan decreased Ang II-induced MCP-1 expression (Fig. 2C). These results suggest that AT₂ receptor stimulation attenuated the AT₁ receptor-mediated increase in NF- κ B binding. We also examined the effect of CGP42112A, an AT₂ receptor agonist, on TNF α -induced NF- κ B binding and observed that CGP42112A effectively inhibited TNF α -induced NF- κ B binding (Fig. 2C).

NF- κ B activation is regulated by I κ B proteins; that is, the dissociation and consequent degradation of I κ B result in an increase in NF- κ B nuclear translocation and its binding to the corresponding *cis* DNA element of the MCP-1 promoter region. Therefore, we first examined the protein levels of cytosolic I κ B after stimulation with Ang II (Fig. 3). Cytosolic extracts were isolated from VSMC, and the levels of

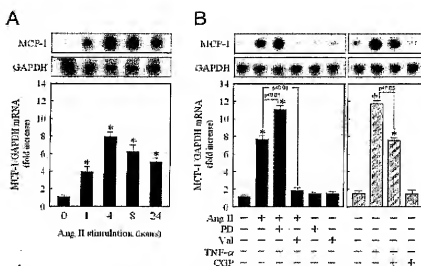


Fig. 1. Expression of MCP-1 mRNA induced by Ang II in fetal rat VSMC

Fetal rat VSMC were cultured as described in *Materials and Methods*. Subconfluent cells were incubated in serum-free DMEM for 24 h before the experiment. MCP-1 mRNA was determined by Northern blot. RNA (25 μ g/lane) was separated by electrophoresis, and hybridized with a probe for MCP-1. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal control. A, Time course of MCP-1 mRNA expression in response to Ang II stimulation. B, Effect of Ang II receptor blockers or CGP42112A on MCP-1 mRNA expression. Total RNA was prepared after 4 h of treatment with Ang II, with or without valsartan or PD123319, or after 4 h of treatment with Ang II, with or without CGP42112A. Values are the mean \pm SE of densitometric measurements ($n = 8$). Similar results were obtained in eight different culture lines. Ang II, 10^{-7} M Ang II; Val, 10^{-6} M valsartan; PD, 10^{-6} M PD123319; TNF- α , 2 ng/ml TNF- α ; CGP, 10^{-7} M CGP42112A. *, $P < 0.05$ vs. control.

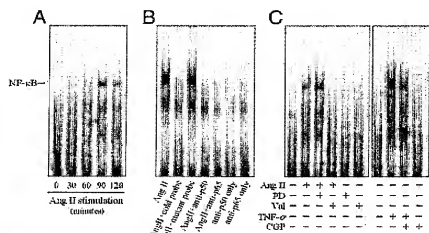


Fig. 2. DNA-Binding Activity of NF- κ B Induced by Ang II

The DNA binding activity of NF- κ B was assayed by EMSA with 32 P-labeled NF- κ B-binding oligonucleotide with nuclear extracts (10 μ g) prepared from fetal VSMC as described in *Materials and Methods*. A, Time course of DNA binding activity of NF- κ B induced by Ang II. B, DNA binding activity of NF- κ B induced by Ang II with unlabeled NF- κ B, mutant NF- κ B, or anti-p50 or anti-p65 antibodies. C, Effect of valsartan, PD123319, or CGP42112A on DNA binding activity of NF- κ B induced by Ang II or TNF- α after 90-min incubation with blockers. Ang II, 10^{-7} M Ang II; Val, 10^{-6} M valsartan; PD, 10^{-6} M PD123319; TNF- α , 2 ng/ml TNF- α ; CGP, 10^{-7} M CGP42112A. Similar results were obtained in seven different culture lines.

I κ B α and I κ B β were determined by Western blot. Upon Ang II stimulation, cytosolic I κ B α and I κ B β levels were decreased, reaching a nadir after 60 min of Ang II stimulation. Simultaneous addition of valsartan attenuated the Ang II-mediated decrease in cytosolic I κ B α and I κ B β levels, whereas addition of

PD123319 exaggerated the decrease in I κ B in the cytosol, suggesting that AT₂ receptor stimulation attenuated the AT₁ receptor-mediated decrease in cytosolic I κ B. TNF- α decreased cytosolic I κ B α and I κ B β levels and CGP42112A attenuated this effect of TNF- α (Fig. 3B).

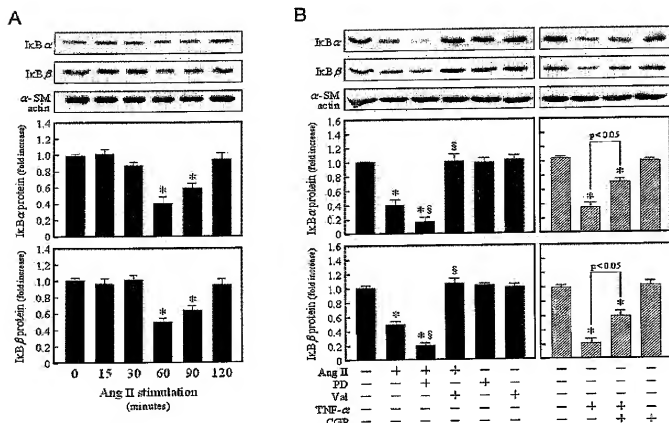


Fig. 3. Regulation of Cytosolic IκB by Ang II in Fetal Rat VSMC

Fetal rat VSMC were cultured as described in Fig. 1. Cytosolic IκB was determined by Western blot using α -SM actin as an internal control. A, Time course of levels of IκB α (upper panel) and IκB β (lower panel) after 60-min stimulation with Ang II. B, Effect of valsartan, PD123319, or CGP42112A on IκB α (upper panel) and IκB β level (lower panel) after 60-min stimulation with Ang II or TNF α . Values are the mean \pm SE of densitometric measurements ($n = 8$). Ang II, 10^{-7} M Ang II; Val, 10^{-5} M valsartan; PD, 10^{-5} M PD123319; TNF α , 2 ng/ml TNF α ; CGP, 10^{-7} M CGP42112A. *, $P < 0.05$ vs. control; †, $P < 0.05$ vs. Ang II.

Roles of AT₂ Receptor-Mediated Activation of Phosphatases in Inhibition of IκB Protein Levels and Attenuation of MCP-1 Expression

To examine the possibility that tyrosine and/or serine/threonine phosphatases are involved in AT₂ receptor-mediated inhibition of MCP-1 expression in fetal VSMC, VSMC were treated with orthovanadate or okadaic acid for 16 h before Ang II addition. As shown in Fig. 4, pretreatment with orthovanadate or okadaic acid further enhanced the Ang II-induced increase in MCP-1 mRNA expression, and these effects were comparable with the effect of AT₂ receptor blockade by PD123319. Pretreatment with orthovanadate or okadaic acid also attenuated the inhibitory effect of AT₂ receptor stimulation by CGP42112A on TNF α -induced MCP-1 expression (Fig. 4). Neither orthovanadate nor okadaic acid affected basal MCP-1 expression. Next, we examined the effects of these phosphatase inhibitors on Ang II-regulated NF- κ B nuclear translocation (Fig. 5). Consistent with the results obtained by EMSA (Fig. 2), nuclear translocation of NF- κ B protein subunits p65 and p50 was enhanced by

specific AT₁ receptor stimulation (Ang II plus PD123319), and Ang II-induced nuclear translocation of NF- κ B was inhibited by selective AT₂ receptor stimulation (Ang II plus valsartan; Fig. 5). Moreover, we observed that both tyrosine and serine/threonine phosphatase inhibitors further increased the Ang II-induced enhancement of NF- κ B protein levels in the nucleus. Addition of these phosphatase inhibitors also enhanced the Ang II-mediated decrease in levels of cytosolic IκB α and IκB β (Fig. 6). The inhibitory effects of CGP42112A on TNF α -mediated nuclear translocation of NF- κ B and the decrease in levels of cytosolic IκB were inhibited by orthovanadate or okadaic acid (Figs. 5 and 6).

Role of SHP-1 in AT₂ Receptor-Mediated Inhibition of MCP-1 Expression

The SH2 domain-containing cytosolic tyrosine phosphatase, SHP-1, has been implicated in the negative regulation of a broad spectrum of growth-promoting receptors. Moreover, we previously demonstrated that AT₂ receptor-mediated activation of SHP-1 was rapid,

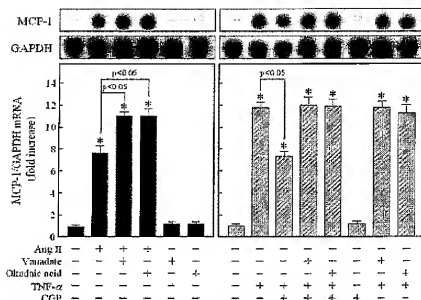


Fig. 4. Effects of Sodium Orthovanadate and Okadaic Acid on MCP-1 Expression Induced by Ang II in Fetal Rat VSMC

Fetal rat VSMC were cultured as described in Fig. 1. Subconfluent cells were incubated with sodium orthovanadate or okadaic acid for 16 h, then stimulated with Ang II or TNF α for 4 h. MCP-1 expression was determined by Northern blot as described in Fig. 1. Values are the mean \pm SE of densitometric measurements ($n = 7$). Ang II, 10^{-7} M Ang II; Val, 10^{-8} M valsartan; PD, 10^{-8} M PD123319; Vanadate, 10^{-5} M sodium orthovanadate; Okadaic acid, 10^{-8} M okadaic acid; TNF α , 2 ng/ml TNF α ; CGP, 10^{-7} M CGP42112A. *, $P < 0.05$ vs. control.

peaking at 2–3 min, and returned to the basal level at 10–15 min, suggesting that SHP-1 is one of the most proximal effectors in AT₂ receptor-mediated apoptosis (8, 11, 15). First, we examined the possibility that AT₂ receptor stimulation may dephosphorylate I κ B and consequently may stabilize I κ B. VSMC were treated with Ang II and immunoprecipitated with anti-I κ B antibodies, and tyrosine phosphorylation of I κ B was examined by immunoblotting analysis with an antiphosphotyrosine antibody, 4G10 (Fig. 7). Tyrosine phosphorylation of I κ B α and I κ B β was enhanced by Ang II and peaked after 10 min of Ang II stimulation (Fig. 7A). Simultaneous addition of valsartan attenuated the Ang II-mediated increase in phosphorylation of I κ B α and I κ B β , whereas addition of PD123319 exaggerated the Ang II-induced tyrosine phosphorylation of I κ B, suggesting that AT₂ receptor stimulation attenuated the AT₁ receptor-mediated tyrosine phosphorylation of I κ B (Fig. 7B). We also examined the effect of CGP42112A on TNF α -induced I κ B phosphorylation and observed that CGP42112A effectively inhibited TNF α -induced phosphorylation of I κ B α and I κ B β (Fig. 7B). To investigate whether SHP-1 plays a role in AT₂ receptor-mediated inhibition of I κ B α and I κ B β degradation, NF- κ B DNA binding activity, and MCP-1 expression, we transfected rat fetal VSMC with a dominant-negative (dn) SHP-1 mutant in which the active cysteine 453 was mutated to serine (C453S) (16). Transfection of the dnSHP-1 mutant was confirmed by immunoblotting, showing a 6-fold increase in SHP-1 immunoreactivity compared with the control vector pcDNA3-transfected VSMC and the decrease

in SHP-1 activity (Fig. 8A). We first examined the functional significance of SHP-1 activation in the AT₂ receptor-mediated inhibition of I κ B α and I κ B β . Basal levels of I κ B α and I κ B β were not changed in either pcDNA3-transfected or dnSHP-1-transfected VSMC. The Ang II-mediated decrease in I κ B α and I κ B β protein levels was more marked in dnSHP-1-transfected VSMC than in pcDNA3-transfected VSMC (Fig. 8B). The Ang II-induced decrease in I κ B α and I κ B β was not significantly influenced by PD123319 in dnSHP-1-transfected VSMC (Fig. 8B). Next we compared the effects of Ang II treatment on NF- κ B binding activity determined by EMSA and MCP-1 mRNA expression in pcDNA3- or dnSHP-1-transfected fetal rat VSMC. In dnSHP-1-transfected VSMC, the Ang II-mediated increases in NF- κ B binding activity and MCP-1 expression were further exaggerated compared with those in pcDNA3-transfected VSMC (Fig. 8, C and D). We also compared the effect of CGP42112A on the TNF α -induced decrease in I κ B α and I κ B β , NF- κ B binding activity, and MCP-1 expression in both cells and found that the effect of CGP42112A was attenuated in dnSHP-1-transfected VSMC (Fig. 8, B–D).

DISCUSSION

Ang II activates an NADH/NADPH oxidase that results in the production of the superoxide anion and, subsequently, hydrogen peroxide (17). The superoxide anion may function as a signaling molecule, mediating in-

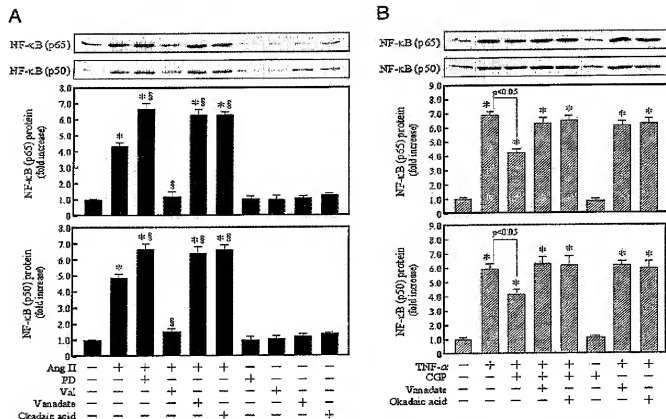


Fig. 5. Effects of Sodium Orthovanadate and Okadaic Acid on Nuclear NF-κB Induced by Ang II in Fetal Rat VSMC

Fetal rat VSMC were cultured as described in Fig. 1. Subconfluent cells were incubated with sodium orthovanadate or okadaic acid for 16 h, then stimulated with Ang II or TNFα for 90 min. NF-κB expression was determined by Western blot using nuclear extract. A, Western blot and densitometric measurements of p50 and p65 subunits of NF-κB. Values are the mean \pm SE of densitometric measurements ($n = 3$). B, Western blot and densitometric measurements of p50 and p65 subunits of NF-κB after treatment with TNFα, with or without CGP42112A and phosphatase inhibitors. Values are the mean \pm SE of densitometric measurements ($n = 4$). Ang II, 10^{-7} M Ang II; Val, 10^{-5} M valsartan; PD, 10^{-5} M PD123319; Vanadate, 10^{-4} M sodium orthovanadate; Okadaic acid, 10^{-5} M okadaic acid; TNF-α, 2 ng/ml TNFα; CGP, 10^{-7} M CGP42112A. *, $P < 0.05$ vs. control; †, $P < 0.05$ vs. Ang II.

creased activity of NF-κB, which coordinates the up-regulation of proinflammatory genes (18). This proinflammatory action of Ang II on the vessel wall interacts synergistically with those of other cardiovascular risk factors, such as dyslipidemia and diabetes. One of the major Ang II-mediated activated proinflammatory genes in monocytes and VSMC is MCP-1, which is inhibited by coadministration of an intracellular antioxidant (19). These Ang II-induced proinflammatory actions in the vessel wall are mediated via AT₁ receptor stimulation. Despite increasing evidence suggesting possible important functions of the AT₂ receptor in vascular remodeling, the specific effect of the AT₂ receptor on vascular inflammation is a total enigma. Consistent with our recent *in vivo* study demonstrating that MCP-1 expression in the injured artery was exaggerated in AT₂ receptor-null mice (4), we observed in this study that AT₂ receptor stimulation antagonized AT₁ receptor-mediated activation of NF-κB, thereby inhibiting MCP-1 expression in cultured fetal VSMC. Moreover, the AT₂ receptor is highly expressed in cer-

tain pathological conditions, such as inflammation and vascular injury (1), and we demonstrated that AT₂ receptor stimulation significantly inhibited TNFα-induced phosphorylation and degradation of IκB, nuclear translocation of NF-κB, NF-κB binding, and MCP-1 expression, suggesting that these inhibitory effects of AT₂ receptor stimulation would contribute to its antiinflammatory actions against other proinflammatory factors as well as AT₁ receptor stimulation. In contrast, Ruiz-Ortega et al. (12) demonstrated that Ang II activates NF-κB via both AT₁ and AT₂ receptors in adult rat VSMC, and Wolf et al. (20) reported that AT₂ receptor stimulation could induce NF-κB activation in AT₂ receptor-expressed COS-7 cells, glomerular endothelial cells, and PC12 cells. These apparent discrepancies could occur because the signaling of these cells may be different from that of the embryonic rat VSMC used in the present study, as the cells used in previous studies were transfected and overexpressed with AT₁ and AT₂ receptors, whereas embryonic VSMC in our study have endogenous AT₁ and

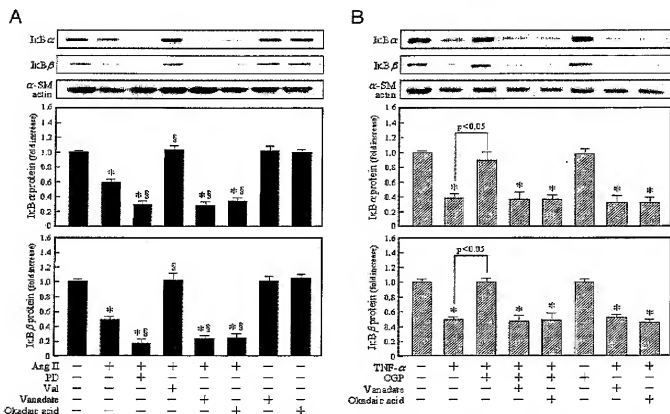


Fig. 6. Effects of Vanadate and Okadaic Acid on IκB Degradation Induced by Ang II in Fetal Rat VSMC

Fetal rat VSMC were cultured, and cytosolic IκBs and α-SM actin were measured as described in Fig. 3. A, Western blot and densitometric measurements of IκBα (upper panel) and IκBβ (lower panel). Values are the mean ± SE of densitometric measurements (n = 4). B, Western blot and densitometric measurements of IκBα (upper panel) and IκBβ (lower panel) after treatment with TNFα with or without CGP42112A and phosphatase inhibitors. Values are the mean ± SE of densitometric measurements (n = 4). Ang II, 10⁻⁷ M; Val, 10⁻⁵ M; PD, 10⁻⁶ M; Vanadate, 10⁻⁶ M; Okadaic acid, 10⁻⁶ M; TNFα, 2 ng/ml; CGP, 10⁻⁷ M CGP42112A. *, P < 0.05 vs. control; †, P < 0.05 vs. Ang II.

AT₂ receptors, and/or could be due to different experimental conditions, such as the concentration of Ang II or the schedule of the reaction.

NF-κB is a critical transcription factor for Ang II-induced MCP-1 gene expression [2, 3, 21]. NF-κB is a heterodimer of p50 and p65 subunits present in an inactive form in the cytoplasm complexed with its inhibitor, IκB. NF-κB activation consists of the dissociation of IκB and nuclear translocation. In this study we observed that Ang II increased nuclear translocation of NF-κB and increased NF-κB DNA binding with a decrease in cytosolic IκBα and IκBβ levels in fetal VSMC, and PD123319 exaggerated these effects of Ang II, suggesting that AT₂ receptor stimulation attenuated AT₁ receptor-mediated NF-κB activation by decreasing IκB levels. Phosphorylation of IκBα and IκBβ dissociates these proteins from NF-κB and determines their fate through degradation. We observed that the addition of orthovanadate and okadaic acid further exaggerated not only the Ang II-mediated increase in MCP-1 expression and NF-κB binding, but also the Ang II-mediated decrease in cytosolic IκB levels to a similar extent when PD123319 was added.

Furthermore, we found that CGP42112A-mediated inhibitory effects on TNFα-induced degradation of IκB, nuclear translocation of NF-κB, and MCP-1 expression were attenuated by the addition of orthovanadate or okadaic acid. These results implied that the inhibitory effect of AT₂ receptor stimulation on AT₁ receptor or TNFα-mediated NF-κB/MCP-1 activation is at least partly due to the phosphatases.

Our results suggest that both tyrosine phosphatase and serine/threonine phosphatase are involved in the AT₂ receptor-mediated inhibition of IκBα and IκBβ degradation. Consistent with our observation, specific serine phosphorylation sites of IκB mediated by IκB kinases (IKKs) and other kinases have been located at two serine residues within their amino-terminal regulatory domains (S32 and S36 in IκBα, and S19 and S23 in IκBβ) [22]. It has also been reported that phosphorylation at the serine of IκBα and IκBβ by serine/threonine kinase triggers polyubiquitination of the IκB proteins [23] and, as with other proteins [24], targets them for rapid degradation by the 26S proteasome. Degradation of IκBα is also mediated by phosphorylation of IκBα at tyrosine residue 42 or by mechanisms involv-

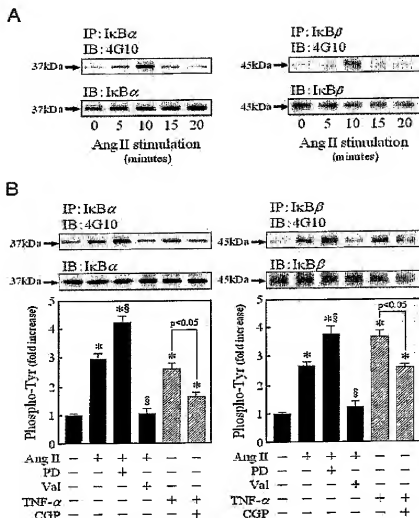


Fig. 7. Effect of Ang II on Phosphorylation of IκB in Fetal Rat VSMC

Fetal rat VSMC were cultured and stimulated with Ang II as described in Fig. 1. Cell lysates were immunoprecipitated with anti-IκBα or anti-IκBβ antibody. Using precipitation, immunoblotting was performed with antiphosphotyrosine antibody 4G10. **A**, Time course of tyrosine phosphorylation of IκBα and IκBβ. **B**, Effects of valsartan, PD123319, and CGP42112A on tyrosine phosphorylation of IκBα and IκBβ. Values are the mean \pm SE of densitometric measurements ($n = 7$). Ang II, 10^{-7} M Ang II; Val, 10^{-6} M valsartan; PD, 10^{-6} M PD123319; Vanadate, 10^{-5} M sodium orthovanadate; Okadaic acid, 10^{-8} M okadaic acid; TNF- α , 2 ng/ml TNF- α ; CGP, 10^{-7} M CGP42112A. *, $P < 0.05$ vs. control; §, $P < 0.05$ vs. Ang II.

ing unidentified protein tyrosine phosphatases (12, 25). It has also been reported that AT₁ receptor-mediated MCP-1 expression was decreased by a tyrosine kinase inhibitor and a MAPK inhibitor (2). Imbart *et al.* (25) reported that stimulation of Jurkat T cells with a protein tyrosine phosphatase inhibitor and the T cell activator, pervanadate, led to NF- κ B activation through tyrosine phosphorylation, but not degradation of IκBα, and that pervanadate-induced IκBα phosphorylation and NF- κ B activation required expression of the T cell tyrosine kinase, p56^{lck}. In addition, inhibition of protein tyrosine phosphatase activities by pervanadate or various vanadate-based compounds has been reported to specifically increase the activation of NF- κ B and downstream responsive genes (26). In this study we showed that both IκBα and IκBβ were

tyrosine-phosphorylated by Ang II stimulation, and this Ang II-induced tyrosine phosphorylation of IκBα and IκBβ was specifically inhibited by an AT₁ receptor blocker, but was enhanced by an AT₂ receptor stimulator, supporting the idea that AT₁ receptor stimulation tyrosine-phosphorylates both IκBα and IκBβ, whereas AT₂ receptor stimulation antagonizes this effect of AT₁ receptor stimulation. Moreover, our results suggest that tyrosine phosphorylation of IκBα and IκBβ is closely associated with degradation of IκBα and IκBβ, and the consequent increase in nuclear translocation of NF- κ B is associated with the enhancement of MCP-1 expression in VSMC.

To examine whether the AT₂ receptor-mediated dephosphorylation of the tyrosine residue is actually involved in Ang II-regulated MCP-1 expression in VSMC,

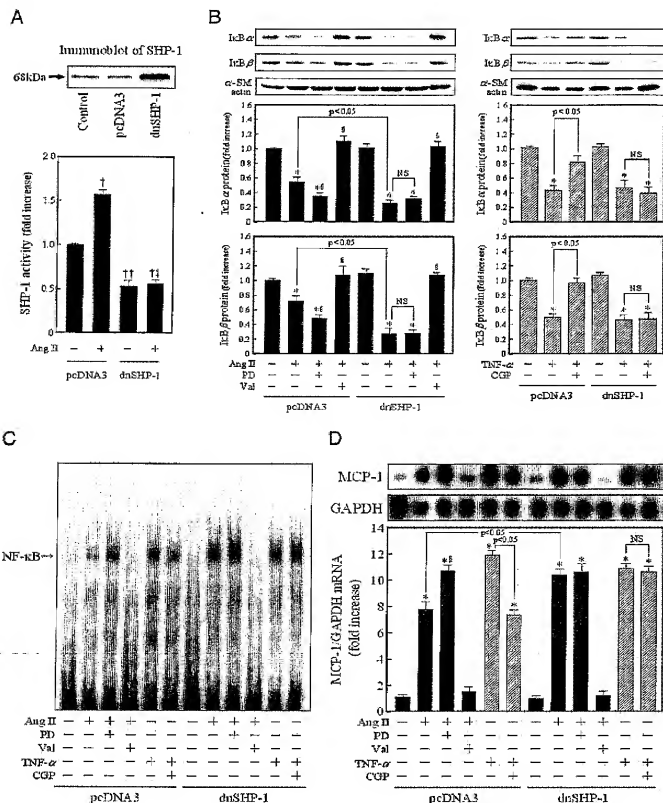


Fig. 8. Effects of dnSHP-1 Transfection on IcB Degradation, NF- κ B DNA-Binding Activity, and MCP-1 Expression Induced by Ang II in Fetal Rat VSMC

Subconfluent rat fetal VSMC were transfected with dnSHP-1 (C453/S) or control vector pcDNA3 as described in *Materials and Methods*. A, Serum-starved cells were stimulated with Ang II for 3 min at 37°C, and cell lysates were assayed for SHP-1 activity. Immunoblot analysis of SHP-1 expression (upper panel) and effect of the transfection of dnSHP-1 on AT₂ receptor-activated

we focused on SHP-1, a soluble tyrosine phosphatase that participates in negative regulation of the receptor tyrosine kinase pathway (27). However, the role of SHP-1 in regulating proinflammatory signaling is incompletely understood. Previously, we demonstrated that SHP-1 is pivotal in the antiangiotensin effect of the AT₂ receptor (8, 11, 15). Moreover, involvement of SHP-1 in AT₂ receptor signaling has been suggested (28). To establish the functional link between AT₂ receptor-mediated activation of SHP-1 and inhibition of the degradation of I κ B, consequent inhibition of NF- κ B binding, and suppression of MCP-1 expression, we employed dnSHP-1. We demonstrated that preventing SHP-1 activation abrogated AT₂ receptor-induced protection of I κ B degradation in fetal VSMC, with a decrease in NF- κ B binding and MCP-1 expression against AT₁ receptor stimulation or TNF α stimulation. Consistent with these results, Massa et al. (29) reported increased sensitivity of astrocytes lacking SHP-1 to various inducers of NF- κ B. These studies suggest an additional role of SHP-1 in controlling specific and nonspecific immune responses where induction of NF- κ B is involved.

It seems possible that SHP-1 could directly dephosphorylate the tyrosine residues of I κ B; however, this possibility requires more extensive examination. On the other hand, there is evidence suggesting that IKKs are themselves phosphorylated and activated by the MAPK cascade (30). Hoshi et al. (31) also reported that PD98059, a MAPK kinase (MEK) inhibitor, suppressed NF- κ B transcriptional activity. MEK kinase-1 has been shown to be copurified with IKK activity (30), and MEK kinase-1 enhances the ability of IKKs to phosphorylate I κ B and activate NF- κ B (32). It is possible that IKKs may be regulated at several levels by SHP-1, as our previous results suggested that SHP-1 is involved in AT₂ receptor-mediated inactivation of ERK (8, 11). Moreover, Shibasaki et al. (28) reported that the AT₂ receptor inhibits epidermal growth factor receptor trans-activation by increasing the association with SHP-1 tyrosine phosphatase and decreases ERK activity. In addition to SHP-1, AT₂ receptor-mediated inhibition of ERK might be dependent on other phosphatases, such as MAPK phosphatase-1 and serine/threonine phosphatase 2A, which results in the inactivation of AT₁- and/or growth factor-activated ERK (1). Consistent with these observations, we demonstrated in this study that inhibition of serine/threonine phosphatases as well as tyrosine phosphatases effectively abrogated the inhibitory effect of the AT₂ receptor-

tor on I κ B phosphorylation, suggesting that the AT₂ receptor-mediated inhibitory pathway of ERK is also involved in this receptor-mediated inhibition of NF- κ B activation.

Ozes et al. (33) reported that the Akt serine-threonine kinase is involved in the activation of NF- κ B by TNF α by activating NF- κ B-inducing kinase, and that Akt mediates IKK α phosphorylation at threonine 23. These findings indicate that Akt is part of a signaling pathway that is necessary for inducing key immune and inflammatory responses. Moreover, Madrid et al. (34) demonstrated that activated phosphoinositide 3-kinase or Akt stimulates NF- κ B-dependent transcription by stimulating trans-activation domain 1 of the p65 subunit rather than by inducing NF- κ B nuclear translocation via I κ B degradation. It has also been reported that upon platelet-derived growth factor stimulation, Akt transiently associates *in vivo* with IKK and induces IKK activation (35). Very recently, we demonstrated that AT₂ receptor-mediated activation of SHP-1 and the consequent inhibition of IRS-2-associated PI-3-K activity contribute at least partly to the inhibition of Akt phosphorylation in PC12W cells (15). These results suggest that AT₂ receptor-activated Akt could be involved in the protective effect of the AT₂ receptor on I κ B degradation and the inhibition of NF- κ B activation and MCP-1 expression.

Impairment of the capacity of a vessel to dilate in the presence of endothelial dysfunction reflects, at least in part, increased oxidative stress due to enhanced catabolism of nitric oxide (NO) caused by increased generation of the superoxide anion. In addition to being a vasodilator, NO is an endogenous inhibitor of VSMC growth and migration (36), of the activity of the transcription factor NF- κ B, and of the expression of proinflammatory molecules (37). With an imbalance between NO and reactive oxygen species (ROS), there is a propensity for vasospasm, VSMC proliferation, prothrombosis, and proinflammatory and prooxidant states. AT₂ receptor-mediated NO production is a well established phenomenon in various tissues and cells (38), and it can be assumed that this NO production might be involved in AT₂ receptor-mediated NF- κ B inactivation. In addition, the possible interesting roles of the AT₂ receptor in NADPH oxidase and production of ROS need to be addressed in the near future to fully understand the molecular and cellular mechanisms of the antiinflammatory effect of AT₂ receptor stimulation.

SHP-1 (lower panel) are shown. Values are the mean \pm SE of densitometric measurements ($n = 3$). B, Western blot and densitometric measurements of I κ B α and I κ B β . Cytosolic I κ Bs were determined by Western blot as described in Fig. 3. Values are the mean \pm SE of densitometric measurements ($n = 4$). C, NF- κ B binding activity determined by EMSA as described in Fig. 2. D, MCP-1 mRNA expression assayed by Northern blot as described in Fig. 1. Values are the mean \pm SE of densitometric measurements ($n = 7$). Ang II, 10^{-7} M; Ang II, Val, 10^{-6} M; valsartan, PD, 10^{-6} M; PD123319; Vanadate, 10^{-6} M; sodium orthovanadate; Okadaic acid, 10^{-6} M; okadaic acid; TNF α , 2 ng/ml; TNF α ; CGP, 10^{-7} M; CGP41212A. \dagger , $P < 0.05$ vs. control, pcDNA3; \ddagger , $P < 0.05$ vs. Ang II, pcDNA3; * , $P < 0.05$ vs. control; \S , $P < 0.05$ vs. Ang II.

Taken together, these results suggest that AT_2 receptor stimulation antagonizes the effect of AT_1 receptor-mediated MCP-1 expression by dephosphorylation of I κ B and consequent inhibition of nuclear translocation of NF- κ B, and that SHP-1 is a critical phosphatase that mediates the antiinflammatory action of the AT_2 receptor. AT_1 receptor-activated NF- κ B also induced proinflammatory genes, such as adhesion molecules, including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, and inflammatory cytokines, such as IL-1, TNF- α , and IL-6, in addition to MCP-1 (39). The potential inhibitory effects of AT_2 receptor stimulation on these proinflammatory genes via NF- κ B inactivation need to be further clarified, and detailed analysis will contribute to further understanding of the pathogenesis of vascular inflammation, atherosclerosis, and vascular remodeling.

MATERIALS AND METHODS

Cell Culture

Fetal VSMC were prepared from the thoracic aorta of Sprague-Dawley rat fetuses (Clea Japan, Inc., Tokyo, Japan; embryonic d 20) and cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum as previously described (40). In addition to morphological observation, the characteristics of VSMC were examined by indirect immunofluorescence using a monoclonal antibody specific for α -smooth muscle (α -SM) actin (clone 1A4; Sigma-Aldrich Corp., St. Louis, MO) (41). Cells at passage 2–3 were used for the experiments. AT_1 and AT_2 receptor binding was measured as previously described (40).

Northern Blot Analysis

Rat fetal VSMC were grown in DMEM with 10% fetal bovine serum to a subconfluent state in 10-cm dishes and were incubated for an additional 24 h in serum-free DMEM to reach a quiescent state. Cells were then treated with vehicle or Ang II (10^{-7} M) with or without valsartan (10^{-5} M; provided by Novartis Pharma AG, Basel, Switzerland) or PD12319 (10^{-5} M; Sigma-Aldrich Corp.). To examine the involvement of phosphatase, cells were incubated with sodium orthovanadate (10^{-5} M vanadate) or okadaic acid (10^{-6} M) for 16 h, then stimulated with Ang II. Recombinant rat TNF- α was purchased from Genzyme (Minneapolis, MN). An AT_2 receptor agonist, CGP42112A, was purchased from Sigma-Aldrich Corp. RNA was prepared from the cells using Trizol reagent (Life Technologies, Inc.). To prepare the hybridization probe for MCP-1, RT-PCR was performed as previously described (4). PCR primers for MCP-1 were 5'-ACT GAA GCC AGC TCT CTC TTC CTC-3' (forward) and 5'-TTC CTT CTT GGG TCA GAG CAG AC-3' (reverse). To verify the identities of the PCR products, we sequenced the PCR products and confirmed that the sequences of PCR products matched the predicted sequences. PCR-amplified DNA of MCP-1 was subcloned into pGEM-T Easy Vector (Promega Corp., Madison, WI). After size fractionation on a denaturing agarose-formaldehyde gel, total RNA (25 μ g) was transferred to a nylon membrane (Hybond-N⁺, Amersham Pharmacia Biotech, Little Chalfont, UK). Hybridization was carried out with 32 P-labeled probes of rat MCP-1 cDNA and/or the 0.78-kb Pst-XbaI fragment of human glyceroldehyde-3-phosphate dehydrogenase in Rapid-Hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was performed us-

ing an image scanner (GT-8000, Epson, Tokyo, Japan) and NIH Image software.

EMSA

NF- κ B DNA binding activity was determined using EMSA as previously described (42). Briefly, after stimulation, the cells were collected with a cell scraper and suspended in buffer A [10 mM HEPES (pH 7.9), 0.5 mM phenethylsulfonyl fluoride (PMSF), 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol (DTT)] and homogenized with a Dounce homogenizer (Kontes Co., Vineland, NJ). Nuclear and cytosolic fractions were separated by centrifugation at $1,000 \times g$ for 15 min at 4°C, and the nuclear fraction was washed twice in buffer A and resuspended in buffer C [20 mM HEPES (pH 7.9), 0.5 mM PMSF, 1.5 mM MgCl₂, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, and 25% (vol/vol) glycerol]. After incubation for 1 h at 4°C, the nuclear fraction was centrifuged at $10,000 \times g$ for 30 min. The supernatant was dialyzed in buffer D [20 mM HEPES (pH 7.9), 0.5 mM PMSF, 0.1 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, and 20% (vol/vol) glycerol] and then cleared by centrifugation and stored at -80°C until use. Specific oligonucleotide probes of NF- κ B rat MCP-1 (5'-GTTC TGG GAA CTT CCA ATG C-3') carrying the rat MCP-1 NF- κ B consensus sequence or its mutation (mutant NF- κ B oligonucleotide, 5'-GTTC TGG GAA CTT CCA ATG C-3'; mutated part is underlined) were prepared as previously reported (43), labeled with [γ - 32 P]deoxy-ATP (3000 Ci/mmol; Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Promega Corp.), and employed to examine the specificity of NF- κ B binding to the rat MCP-1 gene. Nuclear extracts (10 μ g) were equilibrated for 10 min in binding buffer [4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 50 μ g/ml of poly(dI-dC) (Pharmacia Biotech, Uppsala, Sweden)], then the labeled probe (30,000 cpm) was added, and the mixture was incubated for 30 min at room temperature. Competition assays with a 50-fold excess of unlabeled NF- κ B and mutant NF- κ B oligonucleotides were performed to establish the specificity of the reaction. To examine the specificity of binding, 1 μ g anti-p65 or anti-p50 NF- κ B antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added and incubated with nuclear extracts for 1 h before addition of labeled probe. The reaction was stopped by adding gel loading buffer (250 mM Tris-HCl, 0.2% xylene cyanol, and 40% glycerol) and was run on 4% acrylamide gel in Tris-borate-EDTA buffer. The gel was dried and exposed for autoradiography.

Immunoprecipitation and Western Blot Analysis

After stimulation with Ang II, with or without valsartan or PD12319, or stimulation with TNF- α with or without CGP42112A, the cells were quickly washed twice with HEPES-buffered saline and frozen in liquid nitrogen. The cells were lysed in lysis buffer [20 mM HEPES (pH 7.8), 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin, 1 mM Na₂VO₄, and 1 mM NaF], and the supernatant fraction was obtained as the cell lysate by centrifugation at $12,000 \times g$ for 10 min at 4°C. Total cell lysate (300–500 μ g protein) was incubated with 1 μ g anti-I κ B β (Santa Cruz Biotechnology, Inc.) or anti-I κ B α (Santa Cruz Biotechnology, Inc.) at 4°C for 12 h and precipitated by the addition of 25 μ l protein A/G-agarose (Amersham Pharmacia Biotech). The immunoprecipitate was resuspended in 1X Laemmli sample buffer and run on 10% SDS-PAGE. The proteins were then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech), blotted with antiphosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) or anti-I κ B α , I κ B β , p50, p65 (Santa Cruz Biotechnology, Inc.), or α -SM actin antibodies, and detected by an enhanced chemiluminescence method (Amersham Pharma-

cia Biotech). Densitometric analysis was performed using an image scanner (GT-8000, Epson) and NIH image software.

Plasmid Constructs and Transfection

The SHP-1 (C453/S) mutant cDNA was inserted into the pcDNA3 vector (16). Transient transfection was performed with 0.5 μ g plasmid DNA/10-cm dish and Lipofectamine Plus (Life Technologies, Inc.) according to the manufacturer's instructions (DNA:Lipofectamine:Plus ratio, 1:10:10 μ l). After transfection, the transfected cells were fed with complete growth medium for 48 h to allow protein expression. Transfected cells were identified by β -galactosidase staining. The transfection efficiency determined using only the strongly stained cells was $26 \pm 5\%$, similar to that previously reported (11). Overexpression of the SHP-1 (C453/S) mutant was confirmed by immunoblotting with anti-SHP-1 antibody (Santa Cruz Biotechnology, Inc.).

Measurement of Tyrosine Phosphatase SHP-1 Activity

SHP-1 activity was determined as previously described (8). Cell lysates (1 mg protein) were immunoprecipitated with anti-SHP-1 antibody and protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Buckinghamshire, UK). The immunoprecipitates were washed three times with lysis buffer lacking Na_2VO_4 and NaF and once with phosphatase assay buffer [50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 5 mM DTT, and 0.01% Brij35 solution]. Protein tyrosine phosphatase activity was measured as the release of [32 P]orthophosphate from [γ - 32 P]ATP-radiolabeled myelin basic protein.

Statistical Analysis

Results are expressed as the mean \pm SE in the text and figures. Data were analyzed using ANOVA. If a statistically significant effect was found, the data were further analyzed by Dunnett's test to detect the difference between the groups. Statistical significance was assumed for $P < 0.05$.

Acknowledgments

We gratefully acknowledge Novartis Pharma Co. for donating valsartan.

Received February 19, 2003. Accepted December 10, 2003.

Address all correspondence and requests for reprints to: Dr. Masatsugu Horiuchi, Department of Medical Biochemistry, Ehime University School of Medicine, Shigenobu, Osen-gun, Ehime 791-0295, Japan. E-mail: horiuchi@med.ehime-u.ac.jp.

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan; the Japan Research Foundation for Clinical Pharmacology; the Tokyo Biochemistry Research Foundation; and the Smoking Research Foundation.

REFERENCES

- Horiuchi M, Akishita M, Dzau VJ 1999 Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. *Hypertension* 33:613–621
- Chen X-L, Turrmela PE, Olbrich MT, Alexander RW, Medford RM 1999 Angiotensin II induces monocyte/macrophage protein-1 gene expression in rat vascular smooth muscle cells. *Circ Res* 83:952–959

- Usui M, Egashira K, Tomita H, Koyanagi M, Katoh M, Shimokawa H, Takeya M, Yoshimura T, Matsushima K, Takeshita A 2000 Important role of local angiotensin II activity mediated via type 1 receptor in the pathogenesis of cardiovascular inflammatory changes induced by chronic blockade of nitric oxide synthesis in rats. *Circulation* 101:305–310
- Wu L, Iwai M, Nakagami H, Li Z, Chen R, Suzuki J, Akishita M, de Gasparo M, Horiuchi M 2001 Roles of angiotensin II type 2 receptor stimulation associated with selective angiotensin II type 1 receptor blockade with valsartan in the improvement of inflammation-induced vascular injury. *Circulation* 104:2716–2721
- Henkel T, Machiuchi T, Akalay J, Kronke M, Ben-Neriah Y, Baeuerle PA 1993 Rapid proteolysis of I κ B- α is necessary for activation of the transcription factor NF- κ B. *Nature* 365:182–185
- Inagami T, Eguchi S, Numaguchi K, Motley ED, Tang H, Matsumoto T, Yamakawa T 1999 Cross-talk between angiotensin II receptors and the tyrosine kinases and phosphatases. *J Am Soc Nephrol* 10:S57–S61
- Horiuchi M, Lehtonen JYA, Daviet L 1999 Signaling mechanism of AT₂ angiotensin II receptor: cross-talk between AT₁ and AT₂ receptors in cell growth. *Trends Endocrinol Metab* 10:391–395
- Lehtonen JYA, Daviet L, Nahmias C, Horiuchi M, Dzau VJ 1999 Analysis of functional domains of angiotensin II type 2 receptor involved in apoptosis. *Mol Endocrinol* 13:1051–1060
- Yamada T, Horiuchi M, Dzau VJ 1998 The novel angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci USA* 95:156–160
- Shenoy UV, Richards EM, Hang XC, Summers C 1999 Angiotensin II type 2 receptor-mediated apoptosis of cultured neurons from newborn rat brain. *Endocrinology* 140:500–509
- Cui TX, Nakagami H, Iwai M, Takeda Y, Shluchi T, Daviet L, Nahmias C, Horiuchi M 2001 Pivotal role of tyrosine phosphatase SHP-1 in AT₂ receptor-mediated apoptosis in rat fetal vascular smooth muscle cell. *Cardiovasc Res* 49:863–871
- Ruiz-Ortega M, Lorenzo O, Pujol M, Konig S, Wittig B, Eddio J 2000 Angiotensin II activates nuclear transcription factor κ B through AT₁ and AT₂ in vascular smooth muscle cells. *Circ Res* 86:1266–1272
- Wang Z, Castranova MF, Newman WH 2001 NF- κ B is required for TNF- α -directed smooth muscle cell migration. *FEBS Lett* 508:360–364
- Ortega M, Bustos C, Hernandez-Presa MA, Tunon J, Diaz C, Hernandez G, Egido J 1999 Atoxvastatin reduces NF- κ B activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. *Atherosclerosis* 147:253–261
- Cui TX, Nakagami H, Nahmias C, Shluchi T, Takeda M, Matsubara Y, Li J-M, Wu L, Iwai M, Horiuchi M 2002 AT₂ receptor activation inhibits insulin-induced phosphoinositide 3-kinase and Akt, and induces apoptosis in PC12W cells. *Mol Endocrinol* 16:2113–2123
- Lopez F, Esteve JP, Buscail L, Delasque N, Saint-Laurent N, Theveniau M, Nahmias C, Vaysses N, Susini C 1997 The tyrosine phosphatase SHP-1 associates with the set2 somatostatin receptor and is an essential component of set2-mediated inhibitory growth signaling. *J Biol Chem* 272:24448–24454
- Gründling KK, Minieri CA, Ollerenshaw JD, Alexander RW 1994 Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74:1141–1148
- Collins T, Read MA, Nishizawa A, Whitley MZ, Thanos D, Maniatis T 1995 Transcriptional regulation of endothelial cell adhesion molecules: NF- κ B and cytokine-inducible enhancers. *FASEB J* 9:899–909

19. Hernandez-Presa M, Bustos C, Ortego M, Tunon J, Renedo G, Ruiz-Ortega M, Egido J 1997 Angiotensin-converting enzyme inhibition prevents arterial nuclear factor- κ B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation* 95: 1532-1541
20. Wolf G, Wenzel U, Burns KD, Harris RS, Stahl RAK, Thaiss F 2002 Angiotensin II activates nuclear transcription factor- κ B through AT₁ and AT₂ receptor. *Kidney Int* 61:1986-1995
21. Ruiz-Ortega M, Bustos C, Hernandez-Presa MA, Lorenzo O, Plaza JJ, Egido J 1998 Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor- κ B activation and monocyte chemoattractant protein-1. *J Immunol* 161:430-439
22. Karin M 1999 The beginning of the end: I κ B kinase (IKK) and NF- κ B activation. *J Biol Chem* 274:27399-27402
23. DiDonato JA, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S, and Karin M 1996 Mapping of the inducible I κ B phosphorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol* 16:1295-1304
24. Herschko A, Ciechanover A 1992 The ubiquitin system for protein degradation. *Annu Rev Biochem* 61:761-807
25. Imbert V, Ruyter PA, Livolsi A, Paillet JL, Traenklein BM, Mueller-Diekmann C, Farahfar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF 1995 Tyrosine phosphorylation of I κ B α activates NF- κ B without proteolytic degradation of I κ B α . *Cell* 86:787-795
26. Krejci CM, Nadler SG, Esselstyn JM, Kavanagh TJ, Ledbetter JA, Schieven GL 1997 Role of oxidative stress in the action of vanadate phosphotyrosine phosphatase inhibitors. Redox independent activation of NF- κ B. *J Biol Chem* 272:11541-11549
27. Vambatas V, Kaplan DR, Sells MA, Chernoff J 1995 Nerve growth factor stimulates tyrosine phosphorylation and activation of Src homology-containing protein-tyrosine phosphatase 1 in PC12 cells. *J Biol Chem* 270: 25829-25833
28. Shibasaki Y, Matsubara H, Nozawa Y, Mori Y, Masaki H, Kosaki A, Tsutsumi Y, Uchiyama Y, Fujiyama S, Nose A, Iba O, Tateishi E, Hasegawa T, Horiuchi M, Nahmias C, Iwasaka T 2001 Angiotensin II type 2 receptor inhibits epidermal growth factor receptor transactivation by increasing association of SHP-1 tyrosine phosphatase. *Hypertension* 38:667-672
29. Massa PT, Wu C 1998 Increased inducible activation of NF- κ B and responsive genes in astrocytes in the protein tyrosine phosphatase SHP-1. *J Interferon Cytokine Res* 18:499-507
30. Mercurio F, Zhu H, Murray BW, Shlevchenko A, Bennett BL, Li J, Young DBS, Mann M, Manning A, Rao A 1997 IKK-1 and IKK-2: cytokine-activated I κ B kinases essential for NF- κ B activation. *Science* 278:860-866
31. Hoshi S, Goto M, Koyama N, Nomoto K, Tanaka H 2000 Regulation of vascular smooth muscle cell proliferation by nuclear factor- κ B and its inhibitor, I- κ B. *J Biol Chem* 275:883-889
32. Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M 1997 Identification and characterization of an I κ B kinase. *Cell* 90:373-383
33. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB 1999 NF- κ B activation by tumor necrosis factor requires the Akt serine-threonine kinase. *Nature* 401:82-85
34. Romashkova JA, Makatov SS 1999 NF- κ B is a target of Akt in anti-apoptotic PDGF signaling. *Nature* 401:86-89
35. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr, Mayo MW 2000 Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF- κ B. *Mol Cell Biol* 20:1626-1638
36. Dube RK, Jackson EK, Locher TF 1995 Nitric oxide inhibits angiotensin II-induced migration of rat aortic smooth muscle cell: role of cyclic-nucleotides and angiotensin 1 receptors. *J Clin Invest* 95:141-149
37. Peng HB, Libby P, Liao JK 1995 Induction and stabilization of I κ B α by nitric oxide mediates inhibition of NF- κ B. *J Biol Chem* 270:14214-14219
38. Siragy HM, Carey RM 1997 The subtype 2 (AT₂) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest* 100:264-269
39. Kranzhofer R, Browatzki M, Schmidt J, Kubler W 1999 Angiotensin II activates the proinflammatory transcription factor nuclear factor- κ B in human monocytes. *Biochem Biophys Res Commun* 257:826-828
40. Akishita M, Ito M, Lehtonen JYA, Davlet L, Dza VJ, Horiuchi M 1999 Expression of the AT₂ receptor developmentally programs extracellular signal-regulated kinase activity and influences fetal vascular growth. *J Clin Invest* 103:63-71
41. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillesen D, Gabbiani G 1998 A monoclonal antibody against α -smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 103:2787-2796
42. Horiuchi M, Hayashida W, Akishita M, Yamada S, Lehtonen JYA, Tamura K, Davlet L, Chen YE, Hamel M, Cui TX, Iwai M, Minokoshi Y 2000 Interferon- γ induces AT₂ receptor expression in fibroblasts by Jak/STAT pathway and interferon regulatory factor-1. *Circ Res* 86:233-240
43. Wang Y, Rangan GK, Goodwin B, Tay YC, Wang Y, Harris DCH 2000 Lipopolysaccharide-induced MCP-1 gene expression in rat tubular epithelial cells is nuclear factor- κ B dependent. *Kidney Int* 57:2011-2022

